Role of Sit4p-dependent protein dephosphorylation in the regulation of mitochondrial function and yeast lifespan

Andreia Sofia Trindade Pereira

Dissertação de Mestrado apresentada à
Faculdade de Ciências da Universidade do Porto e Instituto de Ciências Biomédicas Abel Salazar

Bioquímica

2014

Role of Sit4-dependent protein dephosphorylation in the regulation of mitochondrial function and yeast lifespan

Andreia Sofia Trindade Pereira
Mestrado em Bioquímica
Departamento de Química e Bioquímica
2014

Orientadores
Doutora Clara Isabel Ferreira Pereira
Instituto de Biologia Molecular e Celular, UP

Professor Doutor Vitor Manuel Vieira da Costa
Instituto de Ciências Biomédicas Abel Salazar, UP
Instituto de Biologia Molecular e Celular, UP

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

Agradecimentos

Um especial agradecimento aos meus orientadores. À Dra. Clara Pereira por todo o conhecimento quer prático quer cientifico transmitido ao longo deste ano. Sem dúvida que a sua disponibilidade, paciência, simpatia e motivação assim como a sua perspicácia e conhecimento científico foram bastante importantes no decorrer deste trabalho. As suas críticas, correções e sugestões fizeram-me crescer quer a nível científico quer pessoal. Ao Prof. Vítor Costa por ter confiado no meu potencial e habilidade dando-me a oportunidade de realizar este trabalho científico no seu laboratório. Os seus conhecimentos científicos assim como a sua orientação foram bastante relevantes no decorrer deste trabalho enriquecendo-o.

Para além dos meus orientadores os meus colegas de trabalho tiveram um papel crucial no decorrer deste trabalho. Sendo assim, queria agradecer a todos os elementos dos grupos RCS, MCA e MicroBiosyn, em especial:

Ao Vítor Teixeira**,** pelas ideias e conversas interessantes nomeadamente com cariz científico. À Vanda Mendes pela sua boa disposição e companheirismo demonstrado durante todo este ano. À Rita Vilaça pela partilha do seu conhecimento em algumas técnicas laboratoriais e também pela transmissão do seu pensamento positivo. Por último, mas não menos importante obrigada Rute Oliveira e Sílvia Píres pelas brincadeiras e conversas que me fizeram sentir rapidamente integrada no laboratório.

Aos meus amigos/familiares por terem entendido as minhas ausências nos programas sociais nomeadamente durante o verão, não é Sofia e Danilo? E também por todo o apoio e compreensão demonstrada durante este ano. Não podia deixar de agradecer em particular ao Daniel, por estar sempre disponível ajudar e por me abrir os olhos quando necessário. Um obrigada muito especial à melhor amiga, não só por estes dois, mas pelos últimos dez anos. Obrigada pela confiança transmitida, por me ouvires naqueles dias mais cinzentos, transformando o meu mau humor e por vezes desilusão naquilo que se chama boa disposição devido ao seu poço inesgotável de amizade.

Ao maninho e cunhadinha, por todos aqueles conselhos sábios durante todo o meu percurso. Por estarem lá sempre que é preciso mesmo quando têm um iceberg de trabalho aglomerado.

Ao Pedro pelo seu grande apoio demonstrado ao longo destes 2 anos e por ter compreendido e aceite a mudança tendo lutado sempre do meu lado, fazendo com que o caminho fosse menos tumultuoso. A tua ajuda e apoio incondicional foram duas peças chave no decorrer desta etapa.

Aos meus pais pelo seu enorme apoio não só financeiro mas também afetivo durante todo o meu percurso académico. Souberam sempre o que dizer naqueles momentos mais complicados e fizeram de mim aquilo que hoje sou.

RESUMO

Nas células, as mitocôndrias têm um papel fundamental na produção de energia, na resistência ao stresse oxidativo e também no envelhecimento. Devido a essas abrangentes funções celulares, as mitocôndrias têm sido alvo de um grande número de estudos na última década. As leveduras têm sido frequentemente usadas como modelo de estudo para desvendar os mecanismos complexos que envolvem estes organelos, devido principalmente à homologia com os organismos superiores.

Em *Saccharomyces cerevisiae*, a proteína Sit4p é uma fosfatase ativada por ceramida, ortóloga à proteína PP6 dos mamíferos, que desempenha um papel fundamental na regulação da função mitocondrial e da longevidade. Na ausência da proteína Sit4p, as células mostram desrepressão catabólica na fase logarítmica (log), resistência ao peróxido de hidrogénio e longevidade aumentada. No entanto, os mecanismos envolvidos nestes processos estão pouco caracterizados. Assim, o principal objetivo deste trabalho consistiu na validação de potenciais alvos mitocondriais da proteína Sit4p com impacto nos fenótipos das células *sit4*Δ. Neste trabalho, foi avaliado essencialmente o papel das proteínas Qcr2p (subunidade 2 da complexo III) e Por1p (um canal aniónico dependente da voltagem da membrana externa da mitocôndria) nos fenótipos das células *sit4*Δ.

Verificamos que os fenótipos das células *sit4*Δ como o consumo de oxigénio na fase log, resistência ao H_2O_2, aumento da longevidade cronológica (LC) e a acumulação de glicogénio são dependentes da proteína Qcr2p. Em relação à Por1p, verificamos que o aumento do consumo de oxigénio e a localização mitocondrial do Isc1p nos mutantes *sit4*Δ são dependentes desta proteína. Numa outra perspetiva verificou-se que a deleção do *SIT4* suprime a sensibilidade ao H_2O_2 e aumentou a LC das células *por1*Δ, estando este fenótipo associado a um aumento da atividade da Cu,Zn-superóxido dismutase (Sod1p). Os mecanismos moleculares que levam à hiperfosforilação da proteína Por1p em células *sit4*Δ também foram abordados neste estudo. Os resultados sugerem que a proteína Sit4p não desfosforila diretamente a Por1p uma vez que não foi encontrada uma interação física entre estas proteínas.

Estes resultados apontam para um potencial envolvimento das proteínas Por1p e Qcr2p nos fenótipos do mutante *sit4*Δ contribuindo assim para o conhecimento da via de sinalização da proteína Sit4 associada à função mitocondrial.

Palavras-chave: função mitocondrial, consumo de oxigénio, stresse oxidativo, longevidade cronológica, Sit4p, Qcr2p e Por1p

ABSTRACT

In cells, mitochondria have a central role in energy production, oxidative stress resistance and also in cell aging. Due to these extensive functions in the cell, these organelles have been the target of a great number of studies in the last decade. Yeast are frequently used as a model organism to unravel the complex mechanisms that involve these organelles, mainly due to the homology to higher organisms.

In *Saccharomyces cerevisiae*, Sit4p is a ceramide activated phosphatase, orthologue of mammalian PP6, which plays a central role in the regulation of mitochondrial fitness and longevity. In the absence of Sit4p, cells show catabolic de-repression in logarithmic (log) phase, resistance to hydrogen peroxide and increased longevity. However, the mechanisms involved in these processes are poorly described. Thus, the main aim of this study was to validate potential mitochondrial targets of Sit4p with impact in *sit4*Δ phenotypes. In this work the role of Qcr2p (subunit 2 of complex III) and Por1p (voltage dependent anion-selective channel of the mitochondrial outer membrane) in *sit4*Δ cells phenotypes was evaluated.

We found that *sit4*Δ phenotypes such as the higher oxygen consumption at logarithmic phase (log phase), resistance to H_2O_2, increased chronological lifespan (CLS) and higher glycogen accumulation are dependent on Qcr2p. Concerning Por1p we verified that the higher oxygen consumption and mitochondrial localization of Isc1p in *sit4*Δ mutant are Por1-dependent. Looking from another perspective, it was found that *SIT4* deletion has the capacity to abolish the H_2O_2 sensitivity and to extend the CLS of *por1*Δ cells, these phenotypes being associated with an increased activity of Cu,Zn-superoxide dismutase (Sod1p). The molecular mechanisms that lead to Por1p hyperphosphorylation in the absence of Sit4p was also addressed in this study. Our results suggest that Sit4p does not seem to directly dephosphorylate Por1p since no physical interaction was found between those proteins.

These data point to a potential involvement of Por1p and Qcr2p in *sit4*Δ mutant phenotypes providing new insights on Sit4p signaling pathways associated with mitochondrial function.

Key-words: mitochondrial function, oxygen consumption, oxidative stress, chronological lifespan, Sit4p, Qcr2p and Por1p

TABLE OF CONTENTS

TABLE LIST

FIGURE LIST

GENERAL ABBREVIATION

2D-gel	Two-dimensional gel
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
CFUs	Colony-forming units
CLS	Chronological lifespan
co-IP	Co-immunoprecipitation
CoQ	Ubiquinone
COX	Cytochrome *c* oxidase
CR	Caloric Restriction
CWI	Cell wall integrity
cyt. C	Cytochrome c
DHS	Dihydrosphingosine
DNA	Deoxyribonucleic acid
DPS	Dipyridyl disulfide
DTT	Dithiothreitol
Duox	Dual oxidase
EDTA	Ethylenediamine tetracetic acid
eIF2α	Eukaryotic translation initiation factor 2
$FADH_2$	Flavin adenine dinucleotide (reduced form)
GFP	Green fluorescent protein
GPX	Glutathione peroxidase
GSH	Glutathione (reduced form)
HIF	Hypoxia-inducible factors
IMAC	Inner membrane anion channel
IMS	Mitochondrial intermembrane space
IP	Immunoprecipitation
IPC	Inositol phosphorylceramide
Isc1p	Inositol phosphosphingolipid phospholipase C
log	Logarithmic
$M(IP)_2C$	Mannosyldiinositol phosphorylceramide
MALDI	Matrix-assisted laser desorption ionization
MIM	Mitochondrial inner membrane
MIPC	Mannosylinositol phosphorylceramide

MOM	Mitochondrial outer membrane
MS	Mass spectrometry
mtDNA	Mitochondrial deoxyribonucleic acid
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate), reduced form
NBT	Nitro blue tetrazolium
Nox	NADPH oxidases
OCR	Oxygen consumption rate
OD_{600}	Optical density at 600 nm
PDS	Post-diauxic phase
PEG	Polyethylene glycol
PHS	Phytosphingosine
phytoCer	Phytoceramide
Pkc1	Protein kinase c 1
Prxs	Peroxiredoxins
RC	Respiratory chain
RET	Reverse electron transport
RLS	Replicative lifespan
RNAs	Ribonucleic acid
ROS	Reactive oxygen species
SAPs	Sit4p associated proteins
SC	Synthetic complete
SL	Sphingolipidis
SNF1	Sucrose nonfermenting protein
SOD	Superoxide dismutase
TCA	Tricarboxylic acid
TEMED	Tetramethylethylenediamine
TOR	Target of rapamycin
TORC1	Target of rapamycin complex I
tRNAs	Transfer ribonucleic acid
Trx	Thioredoxin
VDAC	Voltage dependent anion selective channel
WT	Wild type
YPD	Yeast peptone dextrose
YPGal	Yeast peptone galactose

CHAPTER 1
INTRODUCTION

1.1. Mitochondrial function

1.1.1. General concepts

Mitochondria are ubiquitous organelles present in eukaryotic cells subject of intense investigation over the past 50 years. These organelles are the powerhouse of the cells being their primary function to generate ATP through oxidative phosphorylation (Attardi and Schatz 1988). Beyond this function, mitochondria have a crucial role in redox cell homeostasis, calcium regulation and cell apoptosis and their dysfunction is associated with several diseases in human such as Parkinson's, Alzheimer's, Friedreich's ataxia, multiple sclerosis and amyotrophic lateral sclerosis (Susin, Lorenzo et al. 1999, Duchen 2000, Stewart, Sharpe et al. 2000).

The peculiar characteristics of mitochondria turn these organelles unique. They contain their own genetic information, the mitochondrial DNA (mtDNA), and two membranes (Clayton 1991), sustaining the endossimbiotic theory (Kurland and Andersson 2000) (Figure 1). The mtDNA is a double-stranded circular molecule of 16.5 kb encoding 13 proteins [constituents of the respiratory chain (RC)], 22 transfer RNAs (tRNAs), and 2 ribosomal RNAs (Clayton 1991). The composition of the two membranes and their permeability are quite different. Whereas the mitochondrial outer membrane (MOM) has a lipid to protein ratio of 50:50, the mitochondrial inner membrane (MIM) is more proteinacious (20:80) and contains an unusual phospholipid, cardiolipin. Concerning to their permeability, the MOM is widely permeable to ions and larger molecules due to presence of a voltage dependent anion selective channel (VDAC), while the MIM is much less permeable, having sophisticated ion transporters to allow the passage of specific molecules across this barrier (Krauss 2001, Yamada and Harashima 2008). Due to the existence of these two membranes, mitochondria have four distinct compartments: matrix, MIM, intermembrane membrane space (IMS) and MOM. The mtDNA, ribosomes and the enzymes crucial to the tricarboxylic acid (TCA) cycle and to the fatty acid oxidation are present in the matrix (Figure 1). The inner membrane accommodates the mitochondrial RC that is the main responsible for energy production in cells.

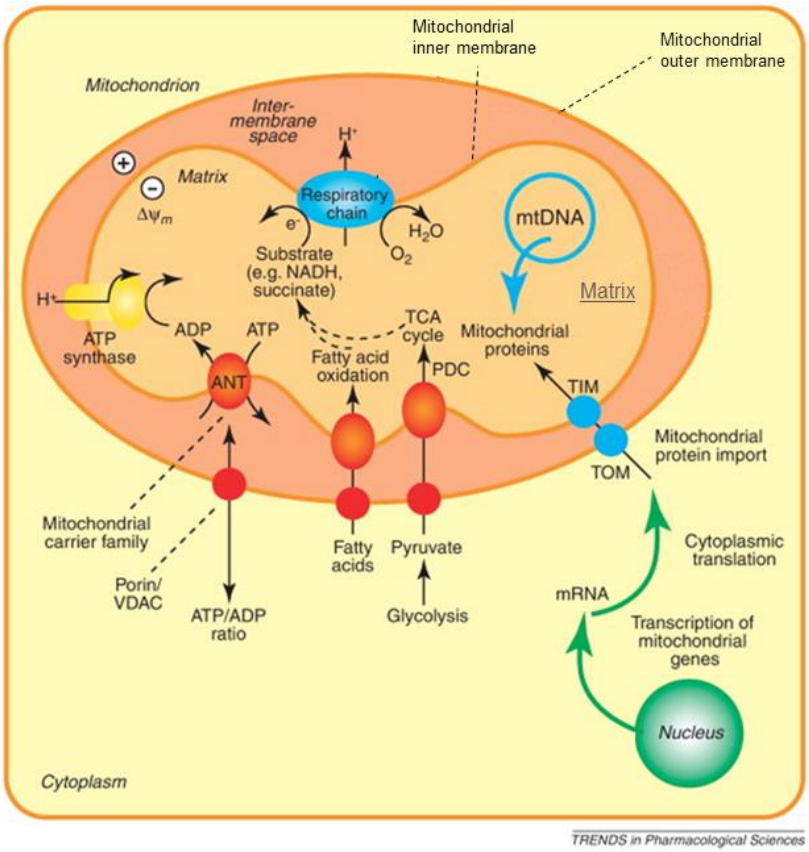

Figure 1 - Mitochondrial function. Mitochondria translocate the metabolites essential for energy production from cytosol through VDAC (MOM) and specific transporters (MIM). Mitochondrial biogenesis requires the coordination among nucleus and mitochondria since mtDNA just codify 13 proteins being the other ones codified by cell genome and translocated to mitochondria through specific machinery. The image was reproduced from (Smith, Hartley et al. 2012). ANT - Adenine nucleotide translocator; mtDNA - DNA mitochondrial; PDC - Pyruvate dehydrogenase complex; TCA - Tricarboxylic acid cycle; TIM - Transporter inner membrane complex; TOM - Transporter outer membrane complex; $\Delta\psi_m$ - mitochondrial membrane potential.

The RC consists in five multi-subunit protein complexes (respiratory complexes I-V), cytochrome c (cyt. c) and ubiquinone (CoQ). Complex I (NADH-ubiquinone oxidoreductase) accepts the electrons from NADH generated during cellular metabolism and transfers them to Complex III (ubiquinone cytochrome c oxidoreductase) via CoQ. Complex III transfers the electrons to Complex IV via cyt. c that reduces oxygen generating water. Accompanying the electron transference, protons are translocated to the mitochondrial intermembrane space creating a proton gradient which provides the energy required for the synthesis of ATP by complex V (F1Fo-ATP synthase) (Mitchell and Moyle 1968). The complex II (succinate-ubiquinone oxidoreductase) has a similar function to complex I but receives the electrons from $FADH_2$ and does not translocate protons to the intermembrane space. As consequence of the RC activity, mitochondria also generate reactive oxygen species (ROS) that contribute to the oxidative stress in the cell (see below).

1.1.2. Mitochondrial generation of reactive oxygen species

ROS are a group of molecules derived from oxygen (O_2) incomplete reduction. Superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$), lipid peroxides (ROOH), ozone (O_3) and hydroxyl radical ($OH^{\bullet}$) are some of the major ROS in living systems.

In the cells there are different organelles that produce ROS such as phagosomes within specialized cells of the immune system used for pathogen killing (Winterbourn 2008), peroxisomes (during fatty acids oxidation) (del Rio, Sandalio et al. 1992), endoplasmic reticulum (during oxidative protein folding and NADH oxidase activity) (Gross, Sevier et al. 2006, Chen, Kirber et al. 2008) and cell membranes [mediated by NADPH oxidases (Nox) and their dual oxidase relatives (Duox)] (Dickinson and Chang 2011). In spite of ROS production in all these sites, mitochondria are still the major ROS generators in cells (Murphy 2009).

The ROS generation by mitochondria occurs during respiration when electrons released from the RC incompletely reduce O_2 to form $O_2^{\bullet-}$ (Chance, Sies et al. 1979). The complex I and III are the two main responsible for this event (Figure 2). As mentioned before, complex I receives the electrons from NADH and normally transfers them to CoQ. However when the RC is blocked (by damage, mutation, ischemia, loss of cytochrome c or by the buildup of NADH due to low ATP demand and consequent low respiration rate) the NADH/NAD^+ ratio increases and complex I reduces directly the O_2 leading to $O_2^{\bullet-}$ formation (Kushnareva, Murphy et al. 2002, Kussmaul and Hirst 2006, Murphy 2009) (Figure 2). During reverse electron transport (RET), complex I also produces large amounts of $O_2^{\bullet-}$ (Adam-Vizi and Chinopoulos 2006, Murphy 2009). This mechanism occurs when electron supply reduces the CoQ pool, which in the presence of high mitochondrial membrane potential forces electrons back from CoQ to complex I, reducing NAD^+ (Figure 2). At last, when the mitochondria are under normal conditions and actively making ATP, the flux of $O_2^{\bullet-}$ from mitochondria is far lower than in the scenarios described before. In this case, the complex III is the main producer of $O_2^{\bullet-}$ being the contribution of complex I negligible (Murphy 2009) (Figure 2). Although less significantly than the respiratory chain, there are others mitochondrial processes that generate ROS such as the TCA cycle and oxidation of palmitoyl-CoA (Murphy 2009).

In order to keep ROS at basal levels, to ensure a normal cell function, organisms contain different antioxidant mechanisms. Some of these defense mechanisms are present in mitochondria and further discussed in the next section.

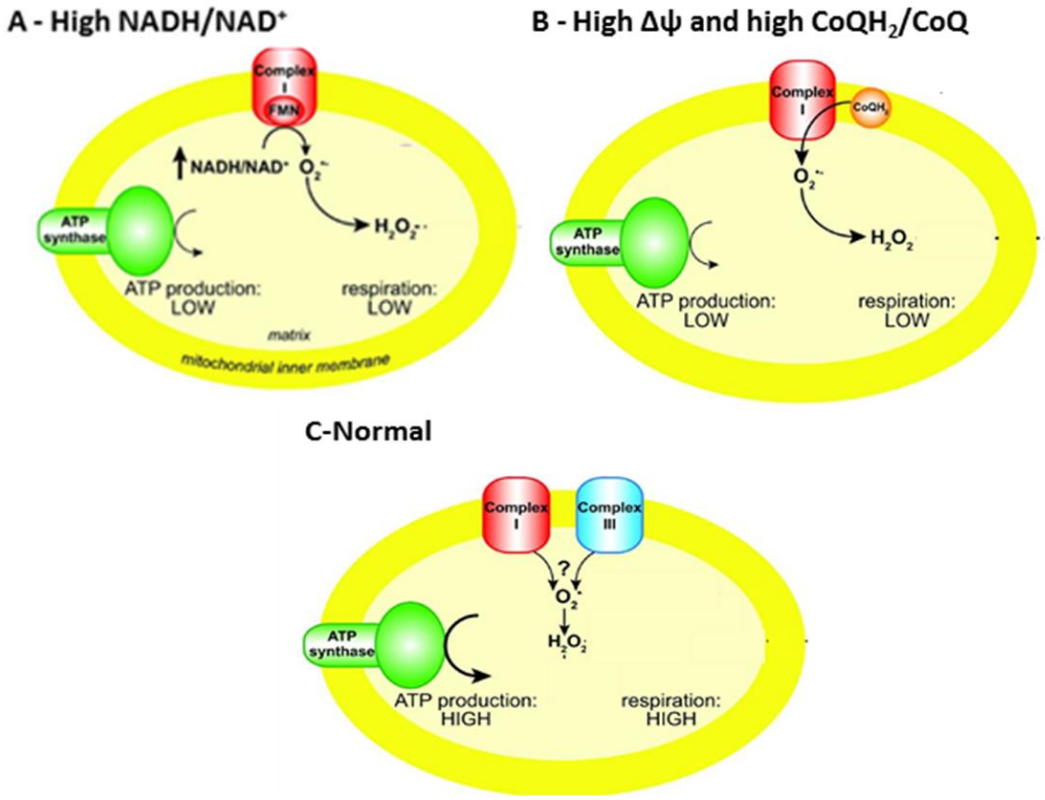

Figure 2 - Models of $O_2^{\cdot-}$ production by mitochondria. A – A high NADH/NAD+ rate, for example promoted by damage at the RC, leads to high production of $O_2^{\cdot-}$ by complex I. B- During the RET the amount of $O_2^{\cdot-}$ is also higher. C- Under normal conditions the $O_2^{\cdot-}$ production is lower and is mainly due to complex III activity. The image was modified from (Murphy 2009). CoQ/CoQH2 - ubiquinone; FMN - Flavin mononucleotide.

1.1.3. Antioxidant systems and redox homeostasis

a) $O_2^{\cdot-}$ detoxification

After $O_2^{\cdot-}$ formation, this radical is rapidly converted into O_2 and H_2O_2 (a less reactive molecule) due the activity of the manganese superoxide dismutase (MnSOD or SOD2) present in the mitochondrial matrix (Fridovich 1995) or by the copper-zinc superoxide dismutase (CuZnSOD or SOD1) present in the mitochondrial intermembrane space and cytosol (Okado-Matsumoto and Fridovich 2001). These enzymes are the first defense line against $O_2^{\cdot-}$, and its importance is demonstrated by the observation that SOD deficiency in mice leads to cardiomyopathy and neurodegeneration (Lebovitz, Zhang et al. 1996) (Figure 3). Other mechanisms of $O_2^{\cdot-}$ detoxification involve its scavenging by α-tocopherol (compound present in mitochondrial membranes) and/or cyt. c (Butler, Jayson et al. 1975, Chow, Ibrahim et al. 1999, Korshunov, Krasnikov et al. 1999).

Some $O_2^{\cdot-}$ produced in the mitochondria may escape from the matrix through the inner membrane anion channel (IMAC) to the intermembrane space and from there to

the cytosol through VDAC (Han, Antunes et al. 2003). For the detoxification of this leaked $O_2^{\bullet-}$ the cytosolic localized SOD1 may play an important role.

b) H_2O_2 detoxification

As mentioned before, one of molecules formed during $O_2^{\bullet-}$ detoxification is H_2O_2 that, despite being less reactive than $O_2^{\bullet-}$, imposes some risks to cell integrity. In mitochondria there are some mechanisms that neutralize H_2O_2 such as the enzyme glutathione peroxidase (GPX), which uses the reduced glutathione (GSH) as substrate to reduce H_2O_2 to H_2O (Panfili, Sandri et al. 1991) (Figure 3). The efficiency of this mechanism depends of GPX activity and GSH concentration (Venditti, Di Stefano et al. 2013). Peroxiredoxins (Prxs) are a group of non-seleno thiol-specific peroxidases that regulate cellular redox homeostasis by reduction of H_2O_2 through the oxidation of thiol-containing proteins such as thioredoxins (Trx) (Rhee, Chae et al. 2005) (Figure 3). Trx and GSH can be reduced by enzymes that use NADPH as a source of reducing equivalents, namely thioredoxin reductase and glutathione reductase, respectively (Handy and Loscalzo 2012).

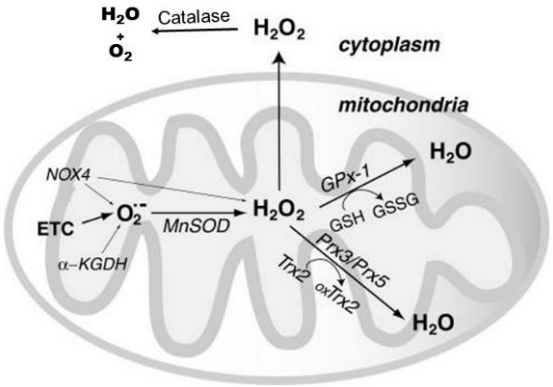

Figure 3 - ROS detoxification mechanism. The image was modified from (Handy and Loscalzo 2012).
GPx 1 - Glutathione peroxidase; GSH/GSSG – Glutathione; MnSOD – Manganese superoxide dismutase; NOX4 - NADPH oxidase 4;Prx2/Prx5 - Peroxiredoxins; Trx2 – Thioredoxins; α-KGDH - α-ketoglutarate dehydrogenase.

Another system for H_2O_2 detoxification is through the catalase activity (Figure 3). Although highly efficient in reducing H_2O_2, catalase may not play a central role in detoxification of H_2O_2 generated in mitochondria since it is mainly localized in the peroxisomes and cytosol. Nevertheless, H_2O_2 can escape from mitochondria to cytosol, due to the high capacity of this molecule to cross membranes, being the catalase activity important in this case (Salvi, Battaglia et al. 2007, Handy and Loscalzo 2012). In addition to catalase, glutathione-GPX and Trx/glutaredoxins systems are also present in the cytosol (Sena and Chandel 2012). At last, H_2O_2 could also react with iron generating $HO^{\bullet}$

by the Fenton reaction (Nakamura, Nakamura et al. 1997). The $HO^{\bullet}$ is a highly reactive molecule promoting several cellular damages.

1.1.4. Cellular effects of ROS

The effect of ROS depends of their amount in cells. For instance, at low concentrations ROS participate in cell signaling (Wink, Nims et al. 1994, Forman, Maiorino et al. 2010, Koopman, Nijtmans et al. 2010), whereas at high concentrations ROS can act as disrupter elements of normal cellular function promoting the damage of cellular lipids, nucleic acids, and proteins (Circu and Aw 2010).

a) Effects of ROS in macromolecules

At a molecular level, ROS have a direct effect on lipids, particularly on phospholipids present in cell membranes, promoting their oxidation into lipid hydroperoxides (Girotti 1998). Other macromolecules that are highly affected by ROS are proteins. Proteins oxidation can be reversible or irreversible, with the first playing an important role in the regulation of signaling pathways (Sena and Chandel 2012). The oxidation of thiol groups is a well-known example of reversible oxidation where H_2O_2 reacts with these groups present in cysteine residues, promoting the generation of disulfide bonds (-SS-), glutathionylation (-SSG) or formation of sulfenyl amides (-SN-). These alterations can lead to intramolecular or intermolecular protein cross-linkages (Sena and Chandel 2012). The irreversible oxidation of proteins, e.g. by nitrosylation of cysteines or carbonylation, can generate aggregates of bulky protein complexes leading to accumulation of damaged proteins and cell death (Stadtman 2004, Poppek and Grune 2006, Valko, Rhodes et al. 2006). The mtDNA is another macromolecule largely affect by ROS, firstly because of the proximity to ROS production place and secondly because of the low capacity of mitochondria to repair mtDNA (Ishikawa, Takenaga et al. 2008). The nuclear DNA is less susceptible to ROS due to the double helix structure and the protective shield of histones and other coating proteins. However, the oxidation of this molecule can still occur and at high scale can induce cell death (Trachootham, Lu et al. 2008).

b) Physiological role of ROS

As mentioned before, ROS also have a physiological role, playing an active part in signaling pathways that regulate cellular processes such as adaptation to hypoxia, autophagy, differentiation, apoptosis and aging (Sena and Chandel 2012).

The family of hypoxia-inducible factors (HIFs) orchestrate the transcriptional response to hypoxia in mammalian cells (Sena and Chandel 2012). The enhanced generation of ROS by mitochondria seems to be crucial to stabilization and induction of HIFα under hypoxia conditions (Brunelle, Bell et al. 2005, Guzy, Hoyos et al. 2005, Mansfield, Guzy et al. 2005), since the pharmacologic or genetic reduction of ROS prevents HIFα induction and impairs the response (Bell, Klimova et al. 2007).

Autophagy is a major catabolic pathway by which eukaryotic cells degrade and recycle macromolecules and organelles (Glick, Barth et al. 2010). This pathway is activated under environmental stress conditions, including oxidative stress and also under starvation. ROS seem to be important both for the induction of macroautophagy (Scherz-Shouval, Shvets et al. 2007) and mitophagy, which mediates the specific elimination of damaged mitochondrial (Qi, Tian et al. 2012).

Stem cells are characterized by the capacity to self-renew, maintaining the stem cell pool, and also differentiate, giving rise to specialized cell (Sena and Chandel 2012). In spite of poorly described, ROS seem to play a significant role in cell differentiation being required for this cellular process, since complex III impairment or treatment with mitochondrial-targeted antioxidants inhibit the process of differentiation (Saretzki, Armstrong et al. 2004, Tormos, Anso et al. 2011, Chaudhari, Ye et al. 2014).

Another crucial role of ROS is in the modulation of apoptosis. The mechanisms of apoptosis are strictly regulated and involve an energy-dependent cascade of molecular events. In the cells, there are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. There is evidence that ROS modulate both these two pathways (reviewed in (Circu and Aw 2010)).

The involvement of ROS in the aging process is widely accepted. In fact, one of the original theories of aging is the free radical theory proposed by Harman (1956). According to this theory, the increase of metabolic rate leads to wide ROS generation by mitochondria leading to cell damage. The accumulation of damages in the cells results in the decline of cellular functions and possibly in organismal death (Harman 1956). However, in recent years this theory was put in doubt leading to the appearance of the mitohormesis theory, which claims that basal ROS production enhance stress resistance

and extends longevity (Pan 2011, Ristow and Schmeisser 2011, Sena and Chandel 2012).

1.2. *Saccharomyces cerevisiae* as an eukaryotic model organism

S. cerevisiae is a unicellular eukaryotic organism belonging to the fungi kingdom. This simpler organism has many cellular pathways conserved being commonly used as model system for the study of basic eukaryotic processes such as DNA repair (Conconi 2008, Aggarwal and Brosh 2010), cell cycle (Hoose, Rawlings et al. 2012), apoptosis (Silva, Saraiva et al. 2012), mitochondrial function, oxidative stress responses and aging (Fabrizio and Longo 2003, Pan 2011, Denoth Lippuner, Julou et al. 2014, Schroeder and Shadel 2014). *S. cerevisiae* has a specific metabolism, when grown in glucose. In a first stage of growth (logarithmic (log) phase) yeast cells obtain energy by fermentation. When glucose is exhausted, cells switch the metabolism to respiratory growth (post-diauxic phase, PDS). The yeast *S. cerevisiae* is a facultative anaerobe and its fermentative capacity allows the survival of cells that lack mitochondrial DNA (rho0) when grown in the presence of glucose.

Beyond the useful informatics tools available for *S. cerevisiae* (genome sequence and databases with complete information about genes and their function in cell) and the easier genetic manipulation, the use of this organism also provides advantages related with the low cost of the culture medium and the shorter generation time when compared to mammalian cells (Mager and Winderickx 2005).

1.3. Yeast Chronological lifespan

Due to the complexity of aging mechanisms in higher organism, as humans, these studies are often performed using model organisms, including *S. cerevisiae.* There are two primary assays for yeast aging, replicative lifespan (RLS) and chronological lifespan (CLS) (Figure 4).

RLS is defined as the number of daughter cells generated by mother yeast cell. These studies have in account that daughter cells are smaller than mothers and can be easily recognized and removed by micromanipulation after budding (Mortimer and Johnston 1959). Yeast replicative aging is considered a model of the aging phenomena observed in asymmetrically dividing cells of higher eukaryotes, such as stem cells (Denoth Lippuner, Julou et al. 2014).

CLS is measured as the time a cell survives in stationary phase, after nutrients become limiting and cells no longer divide (Fabrizio and Longo 2003). These kind of studies mimetic the aging of post-mitotic cells and also as a very simple model of organismal aging (Longo and Fabrizio 2012).

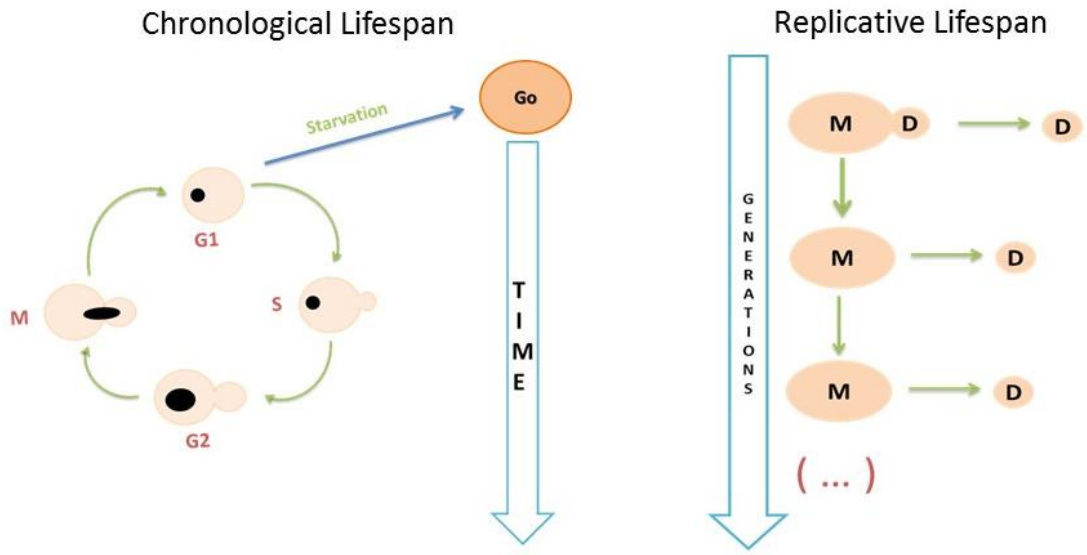

Figure 4 - Schematic representation of chronological and replicative lifespan (Fabrizio and Longo 2003).
D - Daughter cells; M - Mother cell.

As mentioned before, ROS have a central role in regulation of aging mechanisms being the mitochondrial respiratory chain the major producer of these molecules. In yeast there are several signaling pathways that modulate the mitochondrial fitness and oxidative stress responses. The role of nutrients and sphingolipids signaling in the regulation of CLS is described in next section.

1.3.1. Involvement of nutrient signaling in modulation of mitochondrial function and CLS

Nutrient availability in the growth medium modulates yeast signaling pathways that works analogously to growth hormones and mitogens in mammalian cells (Smets, Ghillebert et al. 2010). Upon starvation of essential nutrients, such as carbon source, nitrogen or phosphate, cells cease growth and arrest division, entering into a senescence phase. Nutrient signaling has been associated to modulation of CLS, since caloric restriction (CR) without malnutrition increases lifespan in several organisms (Jiang, Jaruga et al. 2000, Smith, McClure et al. 2007). The target of rapamycin (TOR), the Ras/cAMP/PKA and the sucrose nonfermenting protein (SNF1) pathways, three highly conserved nutrient signaling pathways, seem to be the main players in the extension of CLS under CR conditions (Longo and Fabrizio 2002, Fontana, Partridge et al. 2010).

The TOR pathway is activated in the absence of stress factors by nutrient availability in the growth medium. TORC1 consists of Lst8p, Kog1p, Tco89p and either Tor1p or Tor2p (Loewith and Hall 2011). This complex promotes cell growth, protein synthesis, ribosome biogenesis and amino acids biosynthesis, and inhibits autophagy and stress responses (Loewith and Hall 2011). There are two main effector branches that mediate TORC1 signaling (Loewith and Hall 2011). Sch9p, a member of the AGC kinase family that also includes protein kinase A, protein kinase G, protein kinase C (Pearce, Komander et al. 2010), is the best well characterized substrate of TORC1 (Powers 2007). TORC1 phosphorylates the Sch9p at the C-terminal region in multiple serine and threonine residues, leading to its activation. In the another branch, TORC1 inhibits the activity of type 2A (Pph21p, Pph22p and Pph3p) and type 2A-like (Sit4p, Ppg1p) protein phosphatases by promoting the interaction of these proteins with Tap42p (Di Como and Arndt 1996, Jiang and Broach 1999, Duvel, Santhanam et al. 2003, Zheng and Jiang 2005) (Figure 5).

Down regulation of the TORC1 pathway (either genetically, by CR or rapamycin treatment) increases the CLS of yeast cells (Fabrizio, Pozza et al. 2001, Powers, Kaeberlein et al. 2006). This extended longevity seems to be associated with the induction of autophagic processes, Rim15p activation and promotion of mitohormesis. Autophagy promotes lifespan extension by removing molecules and even whole organelles that are functionally impaired (Lionaki, Markaki et al. 2013). The extension of CLS by reducing TORC1 pathway seems to be partially dependent on Rim15p (Pan, Schroeder et al. 2011) which is a kinase essential to the activation of the Msn2p/Msn4p transcription factors, that control the transcription of stress-responsive genes, and Gis1p,

a transcription factor that drives the expression of PDS element-controlled genes (Gasch, Spellman et al. 2000, Cameroni, Hulo et al. 2004, Roosen, Engelen et al. 2005) (Figure 5). The activation of these transcription factors increases the expression of genes encoding for antioxidant defenses including catalase and SOD that play vital roles in CLS (Fabrizio, Liou et al. 2003). At last, CR and the deletion of *TOR1* or *SCH9* increase respiration and ROS production during log phase, inducing an adaptive response that protects yeast cells against further damage during stationary phase (Mesquita, Weinberger et al. 2010, Pan 2011). This mitohormesis effect seems to have a central role in the regulation of yeast lifespan (Pan 2011, Ocampo, Liu et al. 2012) (Figure 5).

As mentioned above, PKA also plays an important role in the modulation of CLS. The PKA complex is constituted by two of three catalytic subunits (Tpk1p, Tpk2p or Tpk3p) and two regulatory subunits (Bcy1p). PKA activity depends of glucose availably in the growth medium. Beyond the function in promoting ribosome biogenesis, PKA is known for its involvement in the inhibition of stress responses. This complex sequesters the Yak1p kinase in cytosol, preventing the activation of Msn2p transcription factor (Lee, Cho et al. 2008, Malcher, Schladebeck et al. 2011) (Figure 5). Other studies showed that PKA could phosphorylate directly Msn2p, inhibiting the nuclear import of this factor (Gorner, Durchschlag et al. 1998, Garreau, Hasan et al. 2000, Gorner, Durchschlag et al. 2002). Rim15p is another target of PKA and as mentioned before this protein have a crucial role in stress response (Reinders, Burckert et al. 1998). In conclusion, the down regulation of PKA complex culminates with expression of genes associated to stress resistance and mitochondrial fitness leading to extended longevity (Figure 5).

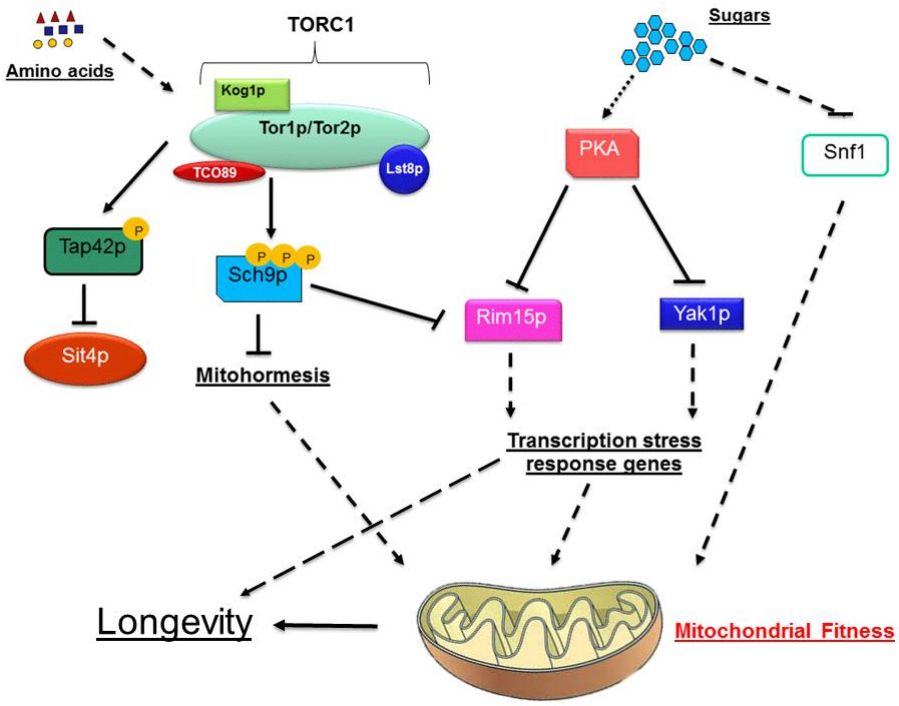

Figure 5 - Integration of nutrient signaling with mitochondrial function and longevity. TORC1 pathway is mainly activated by the amino acids available in the growth medium. This complex has two known direct targets, Tap42p and Sch9p. The phosphorylation of Tap42p culminates with the inactivation of PP2A or PP2A-related proteins, such as Sit4p. TORC1 phosphorylates Sch9p leading to their activation. Sch9p inhibits Rim15p and reduces the mitohormesis effect having a negative contribution in lifespan extension. PKA inhibited Rim15p and Yak1p which are important in transcription of stress response genes. The Snf1 is activated by glucose starvation and promotes the mitochondrial fitness (De Virgilio 2012).

Snf1p, the yeast homolog of mammalian AMP-activated protein kinase (AMPK), is also involved in nutrient signaling. The Snf1p kinase becomes activated under glucose starvation conditions. The SNF1 complex is composed by Snf1p, the catalytic subunit, one β-subunit isoform (Gal83p, Sip1p, or Sip2p), and the Snf4p subunit (Hedbacker and Carlson 2008). Contrarily to what happens in mammalian cells, Snf1p activation by AMP is poorly described but it is known that glucose depletion promotes the access of Snf1p-Thr210 to the activation by its upstream kinases Elm1p, Sak1p and Tos3p (Hong, Leiper et al. 2003, Sutherland, Hawley et al. 2003, Liu, Xu et al. 2011). The phosphorylation of Snf1p at Thr210 is negatively controlled by the Reg1p-Glc7p and Sit4p phosphatases (Ruiz, Xu et al. 2011).

Snf1p controls the gene expression by inhibiting the transcription factor Mig1p (which plays an important role in repress the transcription of genes whose expression is shut off when glucose is present (Carlson 1999)), and stimulating Adr1p, Cat8p and Sip4p (De Virgilio 2012), transcription factors that increase the expression of genes required for the use of non-fermentative carbon sources, gluconeogenesis and

respiration (Hedges, Proft et al. 1995, Lesage, Yang et al. 1996, Young, Dombek et al. 2003). Snf1p activation promotes glycogen storage which is important to fuel the metabolic adaptations to respiratory growth and in the synthesis of trehalose during yeast quiescence (Lillie and Pringle 1980, Eleutherio, Araujo et al. 1993, Francois and Parrou 2001). Another process regulated by Snf1p is autophagy, by a mechanism currently poorly understood (De Virgilio 2012). The precise modulation of Snf1p activity seems to play an important and fine-tuned role in CLS, since both the deletion and the overexpression of *SNF1* decreases yeast lifespan (Lorenz, Cantor et al. 2009, Huang, Liu et al. 2012).

1.3.2. Involvement of sphingolipids in CLS

Sphingolipids (SL) were initially described as structural components of cellular membranes but nowadays is recognized to have broad functions in cellular signaling. These molecules are involved in the modulation of cell cycle, apoptosis and also in the aging process (Epstein, Castillon et al. 2012, Eisenberg and Buttner 2013, Huang, Withers et al. 2014, Montefusco, Matmati et al. 2014). SL have a common structure constituted by a long chain base (LCB), which can be linked to a fatty acid via an amide bond and to a polar head (reviewed in (Rego, Trindade et al. 2013)). In *S. cerevisiae,* the synthesis of sphingolipids starts in the endoplasmic reticulum by condensation of serine with palmitoyl-CoA generating 3-keto dihydrosphingosine (3-keto-DHS) (Hanada 2003). This molecule is reduced to dihydrosphingosine (DHS) by action of NADPH-dependent 3-ketodihydrosphingosine reductase. DHS can be hydroxylated at the C4 position to form phytosphingosine (PHS). Subsequently, the DHS and PHS are acylated generating the dihidroceramide (dhCer) and phytoceramide (phytoCer), respectively. The phytoCer is the central molecule of yeast sphingolipids metabolism, being used as a precursor to form complex sphingolipids by the addiction of polar heads. The phytoCer is firstly converted into inositol phosphorylceramide (IPC) by the transfer of a phosphorylinositol group that is further mannosylated generating mannosylinositol phosphorylceramide (MIPC). Finally, mannosyldiinositol phosphorylceramide ($M(IP)_2C$), the most abundant complex sphingolipid, is formed by addition of another inositolphosphate group (Dickson 2008, Rego, Trindade et al. 2013, Montefusco, Matmati et al. 2014). The hydrolysis of these complex sphingolipids by the action of inositol phosphosphingolipid phospholipase C (Isc1p) releases phytoCer (Matmati and Hannun 2008) (Figure 6).

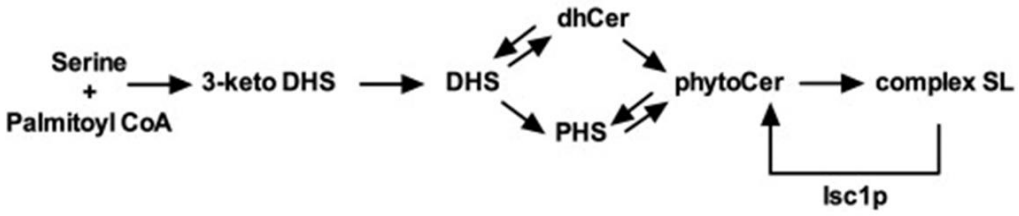

Figure 6 - De novo SL biosynthetic pathway in *S. cerevisiae*. The image was modified from (Spincemaille, Matmati et al. 2014). 3-keto-DHS - 3-keto dihydrosphingosine; dhCer – dihidroceramide; DHS – dihydrosphingosine; Isc1 - inositol phosphosphingolipid phospholipase C; PHS – phytosphingosine.

A recent work showed that down regulation of sphingolipids synthesis by myriocin (inhibitor of SPT) induces an extended longevity in yeast (Huang, Liu et al. 2012). This extended longevity is associated with activation of Snf1p and down regulation of PKA and TORC1, suggesting that myriocin has effects similar to caloric restriction (Liu, Huang et al. 2013). Further support for the involvement of sphingolipids in yeast lifespan is provided by the decreased lifespan upon overexpression of *YDC1*, a gene encoding a ceramidase (Aerts, Zabrocki et al. 2008) or deletion of *ISC1* (Almeida, Marques et al. 2008).

Isc1p localizes mainly in the endoplasmic reticulum of log phase cells, being postranslationally activated by translocation into the mitochondrial outer membrane at late log phase / diauxic shift (Vaena de Avalos, Okamoto et al. 2004, Kitagaki, Cowart et al. 2007). In mitochondria, Isc1p modulates the sphingolipids metabolism since its deficiency leads to an abnormal accumulation of complex sphingolipids in mitochondria and decreased levels of α-hydroxylated phytoceramide (Kitagaki, Cowart et al. 2007). Moreover, *isc1*Δ cells exhibit phenotypes correlated with mitochondrial dysfunction as showed by the high frequency of petite formation, mitochondrial hyperpolarization, fragmentation and low activity of respiratory chain demonstrated by low respiratory rate and decreased cytochrome *c* oxidase (COX) activity (Kitagaki, Cowart et al. 2007, Barbosa, Osorio et al. 2011, Barbosa, Graca et al. 2012, Teixeira, Medeiros et al. 2014). Recent studies, in our lab, showed that the mitochondrial dysfunctions and shorter lifespan of *isc1*Δ cells are associated with the activation of the Sit4p (Barbosa, Osorio et al. 2011).

1.4. Role of Sit4p in yeast cells

Sit4p, the yeast orthologue of mammalian PP6, is a serine threonine phosphatase activated by ceramide (Bastians and Ponstingl 1996). This protein of 35,5 kDa shares 61% amino acid sequence with PP6 and is present in the nucleus and cytoplasm (Bastians and Ponstingl 1996, Huh, Falvo et al. 2003). Initially, Sit4p was described by Arndt, Styles et al. (1989) as a suppressor of *HIS4* transcriptional defect in cells lacking Gcn4p, Bas1p and Bas2p (transcriptional activators of *HIS4* gene).

The involvement of Sit4p in cell cycle progression was also early described by the work of Sutton, Immanuel et al. (1991) where it was showed that the absence of Sit4p leads to cell cycle arrest at late G1 phase being postulated the involvement of Cdc28p, a cyclin-dependent kinase. Cell cycle arrest in *sit4*Δ cells was posteriorly related with Sit4p requirement for normal accumulation of *CLN1*-, *CLN2*- and *HCS26*- mRNA during the late G1 (Fernandez-Sarabia, Sutton et al. 1992). This control of cyclin accumulation by Sit4p occurs at least partly via the Swi4p transcriptional factor (Ogas, Andrews et al. 1991, Fernandez-Sarabia, Sutton et al. 1992).

Sit4p also regulates negatively the protein kinase C (Pkc1p)-cell wall integrity (CWI) pathway (Angeles de la Torre-Ruiz, Torres et al. 2002). This pathway controls important processes in the cell such as yeast budding, cell wall synthesis, cytoskeleton organization and cell cycle (Igual, Johnson et al. 1996, Zarzov, Mazzoni et al. 1996, Ketela, Green et al. 1999, Schmelzle, Helliwell et al. 2002). The Pkc1p-CWI pathway initiates at the plasma membrane through the activation of cell-surface sensors Wsc1/2/3p, Mid2p, and Mtl1p, promoting the association between Rho1p and Pkc1p, which is required for Pkc1p activation by phosphatidylserine (Levin 2011). Pkc1p up-regulation promotes a signaling cascade that culminates with activation of Slt2p/Mpk1p, a mitogen activated protein kinase (MAPK) (Levin 2011). Moreover, the study of Angeles de la Torre-Ruiz, Torres et al. (2002) showed that the cell cycle arrest at G1/S in *sit4*Δ is dependent on Pkc1p but independent on Mpk1p. The mechanism by which Sit4p leads to down regulation of Pkc1 is currently unknown (Angeles de la Torre-Ruiz, Torres et al. 2002) (Figure 7).

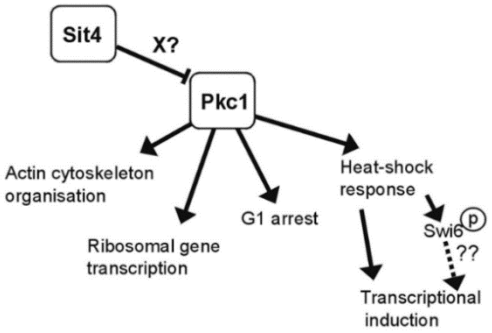

Figure 7 - Schematic model of Sit4p involvement in the modulation Pkc1p-CWI. Sit4p down regulates Pkc1p activity being involved in the regulation of actin cytoskeleton organization, ribosomal gene transcription, cell cycle and cell wall integrity. The mechanism by which Sit4p modulates the Pkc1p activity is unknown, being proposed that Sit4p regulates various elements, which in turn directly interact with Pkc1p. The image was reproduced from (Angeles de la Torre-Ruiz, Torres et al. 2002).

Beyond the involvement of Sit4p in the modulation of Pkc1p-CWI pathway, Singer, Haefner et al. (2003) revealed a functionally link between Sit4p and the ubiquitin-proteasome system. This study showed that *SIT4* mutations when combined with defects in the ubiquitin proteasome promoted by mutations in *PRE1* and *PRE4* (genes that codify β-subunits of the 20S proteasome) are synthetic lethal. However, Sit4p is not a target of proteasome-dependent protein degradation and does not regulate the ubiquitin proteasome pathway, suggesting that these two pathways act on common target proteins.

Sit4p plays also a critical role in telomere function since the telomere length decreases and the silencing of sub-telomere region is lost in cells lacking Sit4p (Hayashi, Nomura et al. 2005). As a consequence, *sit4*Δ cells have a shorter replicative lifespan (Hayashi, Nomura et al. 2005).

Endocytosis is also described to be affected by Sit4p (McCourt, Morgan et al. 2009). The amphiphysin orthologs, Rvs161p and Rvs167p, are proteins important in clathrin-mediated endocytosis and in regulation of membrane dynamics. In their absence endocytosis becomes impaired. However, Sit4p activation by overexpression of *CDC55* (a regulatory subunit of Sit4p) or by loss of *SUR4*, which increases the ceramide levels, restores the endocytic process in cells lacking the amphiphysin-like orthologs (Rvs161 or Rvs167) (McCourt, Morgan et al. 2009). A recent study implicates Sit4p in the regulation of endoplasmic reticulum to Golgi traffic by controlling the direct dephosphorylation of COPII coat subunits (Bhandari, Zhang et al. 2013).

As mentioned above, beyond the activation by ceramide, Sit4p activity is also regulated by TORC1. TORC1 inhibits Sit4p activity by promoting the interaction among

Sit4p and Tap42p (Di Como and Arndt 1996). In response to nitrogen starvation or to TORC1 inhibition, Sit4p dissociates from Tap42p, becoming active. The Sit4p activation promotes the nuclear localization of the Gln3p transcription factor, which induces the expression of transporters and enzymes needed for growth on poor nitrogen sources (Tate, Georis et al. 2010). Sit4p also regulates the activity of Gcn2p, a kinase that regulates the eukaryotic translation initiation factor 2 (eIF2α)(Cherkasova and Hinnebusch 2003). The eIF2α activation leads to the inhibition of protein synthesis and induction of *GCN4* expression, which encodes a transcriptional activator of amino acid biosynthesis (Hinnebusch 2005). Nevertheless, the involvement of Sit4p in eIF2α modulation is not simple, since a direct action of this phosphatase in eIF2α, inhibiting its action, was also described (Cherkasova, Qiu et al. 2010).

The large versatility of Sit4p is closely linked with the different regulatory subunits to which this protein can associate. The interaction of Sit4p with Tap42p, depending on Tap42p phosphorylation by TORC1, results in Sit4p inactivation (Di Como and Arndt 1996). A distinct complex results from the association of Sit4p with the Sit4p associated proteins (SAPs, Sap4p/Sap155p/Sap185p/Sap190p) (Luke, Della Seta et al. 1996). Distinct SAPs can confer different specificities to Sit4p (Luke, Della Seta et al. 1996). A recent study of Woodacre, Lone et al. (2013) showed that Sit4p could also associate to Tpd3p and Cdc55p, in the absence of the SAPs, and this alternative complex was proposed to be involved in the response to ceramide stress.

1.4.1. Sit4p, mitochondrial function and yeast lifespan

In the absence of Sit4p, cells present some particular phenotypes correlated with mitochondrial function such as de-repression of respiration at the logarithmic phase of growth and absence of growth on respiratory substrates such as galactose, ethanol and glycerol (Jablonka, Guzman et al. 2006). The de-repression of respiration at the early phase of growth could be associated with Snf1p activation in *sit4*Δ cells, since Snf1p plays an important role in enhancing the cellular respiration (as described in section 1.3.1.). The low levels of Mig1p, which is involved in the repression of genes whose expression is shut off when glucose is present (Carlson 1999), in *sit4*Δ cells was also suggested as responsible for this phenotype (Jin, Barrientos et al. 2007). The de-repression of respiration at the early phase of growth was recently suggested to contribute to the low fermentative capacity of *sit4*Δ cells (de Assis, Zingali et al. 2013). The low activity of pyruvate decarboxylase observed in this mutant can be due to a feed-back mechanism leading to decreased fermentation since the respiratory chain is active

(de Assis, Zingali et al. 2013). The absence of growth in non-fermentative carbon sources was showed to be associated to deviation of this carbon sources to glycogen accumulation (Jablonka, Guzman et al. 2006).

The *sit4Δ* cells are highly resistant to H_2O_2 and have an extended longevity (Barbosa, Osorio et al. (2011) (Figure 8). These two phenotypes are associated with an increase in antioxidant defenses probably promoted by the active respiration at the log (fermentative) phase. The involvement of Sit4p in mitochondrial function was also recently supported by the observation that *SIT4* deletion reverts defects associated with mitochondrial DNA damage, such as *petite* formation, growth defects and decreased mitochondrial membrane potential (Garipler, Mutlu et al. 2014). This reversion is partially dependent of Snf1p, a downstream target of Sit4p (Garipler, Mutlu et al. 2014).

In spite of the mechanisms by which Sit4p modulates yeast mitochondrial function and lifespan being poorly described, it is possible to correlate some phenotypes with known pathways (Figure 8). Sit4p can be activated by two distinct mechanisms: increase of phytoCer levels or TORC1 inhibition. Sit4p activation impairs mitochondrial function, reduces glycogen accumulation and leads to Snf1p inactivation. Snf1p plays critical roles in glycogen accumulation and also in mitochondrial fitness by modulation of gene expression. These two phenotypes are closely related with yeast longevity. Nevertheless, the mechanism by which Sit4p regulates these process remains unknown but the involvement of Snf1 can be postulated.

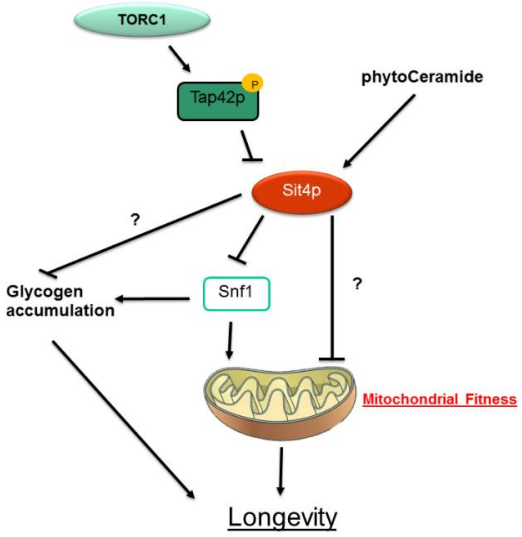

Figure 8 - Involvement of Sit4p in nutrient and sphingolipid pathways. Sit4p is activated by ceramide and inhibited by TORC1. Due to the role of Sit4p in the inhibition of Snf1p, Sit4p activation decreases glycogen accumulation both dependently and independently of Snf1p. Glycogen accumulation in *sit4*Δ cells is associated with extended longevity. Sit4p also modulates mitochondrial fitness by mechanisms poorly characterized that probably involve Snf1p.

CHAPTER 2
AIMS

Mitochondria play a vital role in the regulation of cellular processes such as energy production, stress responses and cell death. Mitochondrial function has also been implicated in aging and age-related disorders in several organisms (Reeve, Krishnan et al. 2008, Karbowski and Neutzner 2012, Bratic, Larsson et al. 2013). Previously studies in our group revealed a crucial role for the phosphatase Sit4p in mitochondrial function and redox homeostasis, impacting longevity (Barbosa, Osorio et al. 2011). Because the mechanism(s) by which Sit4p affects mitochondrial function are unknown, and no mitochondrial targets are described, the aim of this work was to validate potential mitochondrial targets of Sit4p by evaluating their impact in the *sit4*Δ mutant phenotypes. For this purpose, oxygen consumption, oxidative stress resistance, antioxidant enzymes activity and lifespan were analysed in *sit4*Δ cells lacking Qcr2p or Por1p, two of the potential targets previously identified. The overall results will contribute to the characterization of the role of Sit4p in the modulation of mitochondria function and to our understanding of processes associated with aging.

CHAPTER 3
MATERIAL AND METHODS

3.1. Yeast strains and plasmids

The *S. cerevisiae* strains and plasmids used in this study are listed in Table 1.

Table 1 - *Saccharomyces cerevisiae* strains and plasmids used in this study.

Strain	Genotype	Source
S. cerevisiae		
BY4741	Mata, *his3*Δ1, *leu2*Δ0, *met15*Δ0, *ura3*Δ0	EUROSCARF
SIT4-TAP	SC0771 MATa *ade2 arg4 leu2-3,112 trp1-289 ura3-52*	EUROSCARF
APH1-TAP	BY4741 *APH1-TAP*	Yeast-TAP Tagged ORF library
*sit4*Δ	BY4741 *sit4*::*HIS3*	(Vilaça et al, unpublished)
*qcr2*Δ	BY4741 *qcr2*::*KanMx4*	EUROSCARF
*sit4*Δ*qcr2*Δ	BY4741 *sit4*::*HIS3 qcr2*::*KanMx4*	This study
*por1*Δ	BY4741 *por1*::*KanMx4*	This study
*sit4*Δ*por1*Δ	BY4741 *sit4*::*HIS3 por1*::*KanMx4*	This study
i*sc1*Δ	BY4741 *isc1*::*LEU2*	(Barbosa et. al. unpublished)
*sit4*Δi*sc1*Δ	BY4741 *sit4*::KanMx4 isc*1*::*LEU2*	
*por1*Δ*isc1*Δ	BY4741 *por1*::*KanMx4 isc1*::LEU2	This study
*sit4*Δ*por1*Δ*isc1*Δ	BY4741 *sit4*::*HIS3 por1*::*KanMx4 isc1*::*LEU2*	This study
Plasmids		
pYES2-*ISC1-GFP*		(Vaena de Avalos, Okamoto et al. 2004)
pYES2-*ISC1-FLAG*		(Almeida, Marques et al. 2008)
pGAL-*CLbGFP*		(Okamoto, Perlman et al. 1998)

3.2. Genomic DNA isolation

The yeast cells were grown overnight in 10 mL YPD. After that were harvested, washed, pelleted and ressuspended in 100 µL of lysis buffer [2% (vol/vol) Triton X-100, 1% (wt/vol) SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA] and mixed with 100 µL of phenol:chloroform:isoamyl alcohol [50:48:2 (vol/vol/vol)] solution (Harju, Fedosyuk et al. 2004, Hanne; and Xiao 2006). The cells were lysed by vigorous shaking in the presence of glass beads for 3 min. After that, the lysates were centrifuged and the aqueous phase was recovered and added to chloroform. The mixture was homogenized and supplemented with 100 µL TE buffer (100 mM Tris-HCl pH 8.0, 10 mM EDTA). The aqueous phase was recovered by centrifugation and DNA was precipitated with 1 mL of 100% ethanol. The pellet obtained was ressuspended in 400 µL of TE buffer and

incubated with 30 µg RNAse for 5 min at 37°C. The reaction was stopped by the addition of 10 µL of ammonium acetate and the DNA precipitated by addition of 1 mL of 100% ethanol. The pellet was washed twice with 70% (vol/vol) ethanol, dried and ressuspended in water. The genomic DNA was quantified using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific).

3.3. Polymerase Chain Reaction (PCR) procedure

The PCR programs used in this study are described in Table 2. The reaction mix used in each reaction contained 1 x Reaction Buffer (Thermo Scientific), 1.5 mM $MgCl_2$ (Thermo Scientific), 0.2 mM sense primer, 0.2 mM antisense primer, 0.2 µM dNTPs (Thermo Scientific), 1 U Taq Polymerase (Thermo Scientific) and 300 ng genomic DNA for a final volume of 20 µL.

3.4. DNA manipulation and plasmids

Null mutations of *POR1*, *SIT4*, and *ISC1* were created by homologous gene replacement using DNA fragments generated by PCR. The deletion fragment containing *KanMX4* and the flanking regions of *POR1* were amplified from genomic DNA of the W303 *por1*::*KanMx4* strain using the primers POR1_Amp_Fw and POR1_Amp_Rv (listed in Table 2).

The *HIS3 and LEU2* cassettes for deletion of *SIT4* and *ISC1*, respectively, in the *por1*Δ background, were amplified from BY4741 *sit4*::*HIS3,* BY4741 *isc1*::*LEU2* as described above (Table 2).

All the PCR products were analysed by nucleic acid electrophoresis (using TAE agarose gel). The purification of the PCR products was performed with the Gel Band Purification Kit (GE Healthcare). The yeast cells were transformed using the LiAc/SS carrier DNA/PEG, as described below. The cells were selected in SC medium lacking leucine or histidine or in YPD supplemented with geneticin, depending on the selection cassette.

For the analysis of Isc1p mitochondrial recruitment in the different strains, yeast cells were transformed with a plasmid expressing Isc1p-GFP (pYes2-*ISC1-GFP*) and selected in minimal medium lacking uracil.

For the evaluation of the mitochondrial network, yeast cells were transformed with plasmid CLbGFP.

Table 2 - **Primers and PCR programs used in this work**

Gene	Primers	PCR program	
		Annealing temperature (ºC)	**Elongation time (min)**
por1::*KanMX4*	**POR1_Amp_Fw** AGTTTAATGGTCAGAATGGGCG **POR1_Amp_Rv** GGAGTTTATCACAATGTTCGAAACC	51	2.50
sit4::*HIS3*	**SIT4_Amp_Fw** TATTGAAGCTCAAAAACATCCATAATAAAAGGAA CAATAACAATG **SIT4_Amp_Rv** AATTATTTTTATTCGTCGAGTTAGGGAGGGCATG CCGTCGTGTTA	55	1.50
isc1::*LEU2*	**ISC1_Amp_Fw** CTTTCCGCGTAAAAAGGGAA **ISC1_Amp_Rv** TTGCTTTGCATCTATTGACGA	50	2

3.5. Yeast transformation

For yeast transformation, the PEG/lithium acetate method was used (Gietz and Schiestl 2007). Cells were grown in 20 mL of YPD medium since an OD_{600}= 0.2 to OD_{600}= 0.8. After that, the cells were harvested, washed and incubated with the transformation mix, containing 240 µL of Polyethylene Glycol 3350 (PEG 3350) 50% (w/v), 36 µL Lithium Acetate 1.0 M, 25 µL Single-stranded carrier DNA (2.0 mg ml^{-1}) and 59 µL DNA plus sterile water. In case of plasmid transformation, the cells were incubated at 42ºC for 30 min, whereas for gene deletion the cells were initially incubated at 26ºC for 30 min and afterwards incubated at 42ºC for 30 min. Lastly, the cells were centrifuged, washed and plated on selective medium. A negative control containing the cells and transformation mix was always used.

3.6. Growth conditions

The yeast cells were grown aerobically at 26°C in a gyratory shaker at 140 rpm, with a ratio of flask volume/medium volume of 5:1, to logarithmic (log) phase (OD_{600}=0.6) or to post-diauxic (PDS) phase (OD_{600}=7-10). The growth media used were yeast peptone dextrose [YPD; 1% (wt/vol) yeast extract, 2% (wt/vol) bactopeptone, 2% (wt/vol) glucose], yeast peptone galactose [(YPGal; 1% (wt/vol) yeast extract, 2% (wt/vol) bactopeptone, 2% (wt/vol) galactose)] and synthetic complete [SC medium; containing drop-out, 2% (wt/vol) glucose or 2% (wt/vol) galactose and 0.67% (wt/vol) yeast nitrogen

base without amino acids (BD BioSciences)] and supplemented with appropriate amino acids or nucleotides (0.008% (wt/vol) histidine, 0.008% (wt/vol) tryptophan, 0.04% (wt/vol) leucine and 0.008% (wt/vol) uracil).

3.7. Co-immunoprecipitation

3.7.1. Sit4p-TAP

Total protein extracts were prepared from 10 mL cultures in PDS of cells expressing Sit4p-TAP or Aph1p-TAP. The cells were harvested, washed and the pellets were ressuspended in the co-immunoprecipitation (co-IP) buffer A [50 mM Tris-HCl pH 7.3, 0.1% (vol/vol) Triton X-100, 10% (vol/vol) glycerol, 100 mM NaCl, 50mM sodium fluoride, 1 mM DTT, 1X Complete Mini Protease Inhibitor Cocktail (Roche)]. After that, the cells were lysed by vigorous shaking of the cell suspension in the presence of zirconia beads for 8 min (short pulses of 1 min with 1 min intervals on ice). The soluble protein was separated from the cell debris by centrifugation at 13 000 r.p.m. for 15 min and the protein concentration was assessed by the Lowry method using bovine serum albumin as a standard. After that, 3 mg of protein were incubated with 25 µL of Rabbit IgG−Agarose (A2909 Sigma) (which recognized TAP) for 2 h at 4°C. The unbound proteins were removed by three washing steps with 1mL co-IP buffer A. Finally, the proteins complexes were eluted with 60µL of Laemmli sample buffer [125 mM Tris HCl pH 6.8, 4% (wt/vol) SDS, 20% (vol/vol) glycerol, 10% (vol/vol) 2-mercaptoethanol, 0.004% (wt/vol) bromophenol blue] and the beads were removed by centrifugation. The supernatant was analysed by SDS-PAGE and western blot, as described below.

3.7.2. Isc1p-Flag

Cells expressing the Isc1p-Flag were grown to OD_{600}=5 in 10 mL of SC medium lacking uracil, using galactose as carbon source. The cells were harvested, washed and the pellets were ressuspended in the co-IP buffer B [0.5% (vol/vol) Triton X-100, 150 mM NaCl, 1 mM EDTA, 50 mM Tris pH 7.4, 50mM sodium fluoride, 1X Complete Mini Protease Inhibitor Cocktail (Roche)]. The cells were lysed and total protein quantified as described before. The protein extract (3 mg) was incubated at 4ºC during 2 h with an antibody that recognizes the FLAG (F3165 Sigma). After that, Protein G Sepharose (P3296 Sigma) was added and the mix was incubated during 1 hour. The unbound proteins were removed by three washing steps with 1mL co-IP buffer B. The protein complexes were eluted as described before and analysed by SDS-PAGE and western blot, as described in next section.

3.8. Western blot analysis and stripping for reprobing

Proteins were separated using a 12% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking with low fat dry milk, membranes were probed overnight with the primary antibodies, anti-TAP (1:30 000, F7 Santa Cruz), anti-FLAG (1:10 000, F3165 Sigma) or anti-por1 (1:4000, Invitrogen A6449), followed by a 2 h incubation with the correspondent secondary antibodies conjugated to horseradish peroxidase, anti-rabbit IgG light chain (1:10 000, ab99697) or anti-mouse (1:5000, Invitrogen G21040).

The membrane stripping was performed as described in the Abcam protocol. The membrane was washed with TTBS, to remove the ECL remained, and was incubated with a stripping buffer (62.5 mM Tris-HCL pH 6.8, 2% (wt/vol) SDS, 100 mM 2-mercaptoethanol), previously warmed, for 30 min at 50ºC. After that, the membrane was washed several times to eliminate all the 2-mercaptoethanol.

3.9. Oxygen consumption

The oxygen consumption rate (OCR) was measured in whole yeast cells grown to log phase or PDS phase, using a Clark oxygen electrode. Cells (3×10^8) were collected, ressuspended in 1 ml PBS buffer (137 mM NaCl pH 7.4, 2.7 mM KCl, 8 mM Na_2HPO_4 and 1.46 mM KH_2PO_4) being add to the water-jacketed microcell, magnetically stirred, at 26°C. The OCR was measured for 5 min using an Oxygraph system (Hansatech) and data analysed with the Oxyg32 V2.25 software (Hansatech).

3.10. Hydrogen peroxide resistance and chronological lifespan

For the analysis of hydrogen peroxide resistance, yeast cells were grown in YPD to log or PDS phase and treated with 2.5 mM or 250 mM H_2O_2 (Merck), respectively, for 30 min. Chronological lifespan (CLS) was assayed as described by Vilaca, Silva et al. (2014). Briefly, overnight cultures in SC medium were diluted to OD_{600}=0.6, grown for 48 h (stationary phase, considered t0 in lifespan assay) and maintained in culture media for the indicated time. Cell viability was determined by standard dilution plate counts on YPD medium containing 2% agar (wt/vol). Colonies were counted after growth at 26ºC for 3

days in plate. Cellular viability was expressed as the percentage of the colony-forming units (CFUs) (treated cells versus non-treated with H_2O_2 cells or in relation of t0 in CLS).

3.11. Determination of glycogen content

Glycogen content was measured qualitatively by exposition of yeast colonies to iodine vapor. Cells were grown for 3 days in liquid medium, followed by 4 days growth in YPD plates. Plates were then inverted over a monolayer of iodine crystals for 5 min (Yang, Chun et al. 1998). The yeast colonies develop a brown color, with the darker the staining, the greater the glycogen accumulation.

3.12. Fluorescence microscopy

In order to evaluate, in each mutant, the recruitment of Isc1p to mitochondria, yeast cells carrying a plasmid that expresses the Isc1p-GFP fusion protein (pYes2-*ISC1-GFP*) were grown in YPGal to OD_{600}=2. The MitoTracker Red CM-H_2XRos probe was used to stain the mitochondria. Live cells were observed by fluorescence microscope (AxioImager Z1, Carl Zeiss). The counts were processed taking in account the typical morphology of endoplasmic reticulum and mitochondria.

The mitochondrial network was visualized in cells expressing a matrix-targeted GFP. The cells were grown to OD_{600}=2 in YPGal medium and were analysed using a AxioImager Z1 (Carl Zeiss) fluorescence microscope.

3.13. Catalase activity

Catalase activity was evaluated in yeast cultures grown to PDS phase. The cells were lysed in 50 mM potassium phosphate buffer (pH 7.0) containing protease inhibitors (Complete, Mini, EDTA-free Protease Cocktail Inhibitor Tablets; Boehringer Mannhein) by vigorous shaking of the cell suspension in the presence of zirconia beads for 8 min (short pulses of 1 min with 1 min intervals on ice). Protein concentration in cellular extracts was assessed as described above. Catalase activity was analysed spectrophotometrically as described previously by Aebi (1984). Briefly, 100 µg of protein was diluted in 50 mM potassium phosphate buffer (pH 7.0) and H_2O_2 added at a final concentration of 10 mM in 1 mL of reaction. The absorbance variation per min at 240 nm was measured at 25ºC, using the spectrometer Shimadzu UV-2401 PC (Shimadzu Corporation).

3.14. Superoxide dismutase activity

The superoxide dismutase activity was determined *in situ*, as described by Flohe and Otting (1984). The cells were grown to PDS phase and the proteins were extracted and quantified as described above. To evaluated the Sod1p and Sod2p activity 25 µg and 100 µg of the protein extracts, respectively, were separated on a native 10% polyacrylamide gel and after that, the gel was incubated sequentially with 2.5 mM nitro blue tetrazolium (NBT) for 20 min and with development solution (36 mM potassium phosphate pH 7.8, 28 mM TEMED and 86 µM riboflavin) for 15 min in the dark. The color was then developed by exposure to light. To measure Sod2p activity, Sod1p was inhibited by addition of 2 mM KCN to the development solution. The SOD activity was assessed by band intensity quantification. To evaluate the quantity of protein present in each lane, the gels were staining with Brilliant Blue G-colloidal Concentrate (B2025, Sigma) using the suppliers protocol. Briefly, after an additional destaining step with 10% (vol/vol) acetic acid in 25% (vol/vol) methanol during 1 h, the proteins were fixed for 30 min with a solution of 7% (vol/vol) acetic acid in 40% (vol/vol) methanol. Afterward the gel was stained using a mix of stain solution with methanol (4:1) during 1 h. Finally, the gel was destained with 10% (vol/vol) acetic acid in 25% (vol/vol) methanol for 1 min and rinsed in 25% (vol/vol) methanol for 24 h.

3.15. Statistical analysis

Data were analysed in GraphPad Prism Software v6.02 (GraphPad Software) and expressed as mean values ± SD from at least three independent experiments. Values were compared by one-way ANOVA *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$; ****, $p<0.0001$.

CHAPTER 4
RESULTS

4.1. Mitochondrial phosphoproteome of *sit4*Δ cells

Several studies support a crucial role of Sit4p in catabolic repression, resistance to oxidative stress and in longevity of yeast cells (Jablonka, Guzman et al. 2006, Barbosa, Osorio et al. 2011). Due to Sit4p role in mitochondrial modulation, it was postulated that Sit4p, as phosphatase, alters the mitochondrial phosphorylation pattern leading to its dysfunction.

In order to test this hypothesis, the mitochondrial phosphoproteome of the WT and *sit4*Δ cells was assessed by western-blot of 2-dimensional gels (2D-gel) using an antibody that recognize phosphoresidues (C. Pereira et al., unpublished) (Chapter 8 Figure S1). Proteins differentially phosphorylated in *sit4*Δ cells were identified by mass spectrometry (MALDI-MS/MS), using spots cut from a silver stained 2D-gel replicate (C. Pereira et al., unpublished) (Chapter 8 Table S1). These analyses showed that when comparing to WT, *sit4*Δ mutant has 9 mitochondrial proteins hyperphosphorylated which are associated with distinct functions, such as the respiratory chain (Qcr2p, Atp1p and Atp2p), the passage of metabolites to the mitochondria (Por1p), antioxidant defense (Sod2p), amino acid synthesis (Ilv5) and heat shock response (Hsp26p, Ssc1p) (Table S1).

In this study, we evaluated the role of these potential Sit4p targets, namely Qcr2p and Por1p, in the phenotypes of *sit4*Δ cells. Qcr2p is the subunit 2 of ubiquinol cytochrome-c reductase (Complex III) (Schoppink, Hemrika et al. 1989). Por1p is a voltage selective channel that allows the passage of metabolites between cytoplasm and mitochondrial intermembrane space (IMS) (Lee, Xu et al. 1998).

The MALDI-MS/MS analysis of Qcr2p found six peptides potentially phosphorylated which contain fifteen phosphorylatable residues (Ser/Thr/Tyr) (Table 3). However the identification of the individual phosphorylated residues was not possible since the amount of phosphorylated peptides was not sufficient to allow protein sequencing. Enrichment in the phosphorylated peptides will be required to analyse the Qcr2p phosphorylated residues in *sit4*Δ cells.

Table 3 - Qcr2p phosphopeptides potential regulated by Sit4p

Sequence of the phosphorylated peptides (potentially phosphorylated residues in bold)	Phosphorylatable amino acid
I**ST**LAVK (28-34)	S29 T30
VHGG**S**R**Y**A**T**KDGVAHLLNR (35-53)	S39 Y41 T43
TAFKPHEL**T**E**S**VLPAAR (112-128)	T112 T120 S122
GLGNPLL**Y**DGVERV**S**LQDIKDFADK (154-178)	Y161 S168
LDKF**T**DGGLF**T**LFVR (276-290)	T280 T286
NAVQNV**S**V**SS**PIELNFDAVK (325-344)	S331 S333 S334

In the case of Por1p, the MALDI-MS/MS analysis showed that in cells lacking Sit4p, Por1p is phosphorylated on 2 novel amino acid residues (Ser133 and Ser261), which are not conserved in human Por1p. In order to identify the localization of these phosphorylated residues, they were mapped onto the 3D x-ray crystallographic structure of human Por1p, considering the alignment of Por1p sequence from *S. cerevisiae* and human (Figure 9). Based on this model was found that Ser133 is localized in cytosolic loop while Ser261 is localized in a β-sheet with the R group turned to the pore (Figure 9, Table 4).

Moreover, using a bioinformatic tool, the kinases that potential phosphorylate these residues were assessed. NetworKIN analysis resulted in p38MAPK (human homolog of Hog1p), caM-II (homologous of CaM kinase), GSK3 (Rim11p in yeast) and CDK5 (human homolog of Pho85p) as potential kinases for Ser163 phosphorylation. In the case of Ser261, the CKII was the predicted kinase (Blom, Gammeltoft et al. 1999).

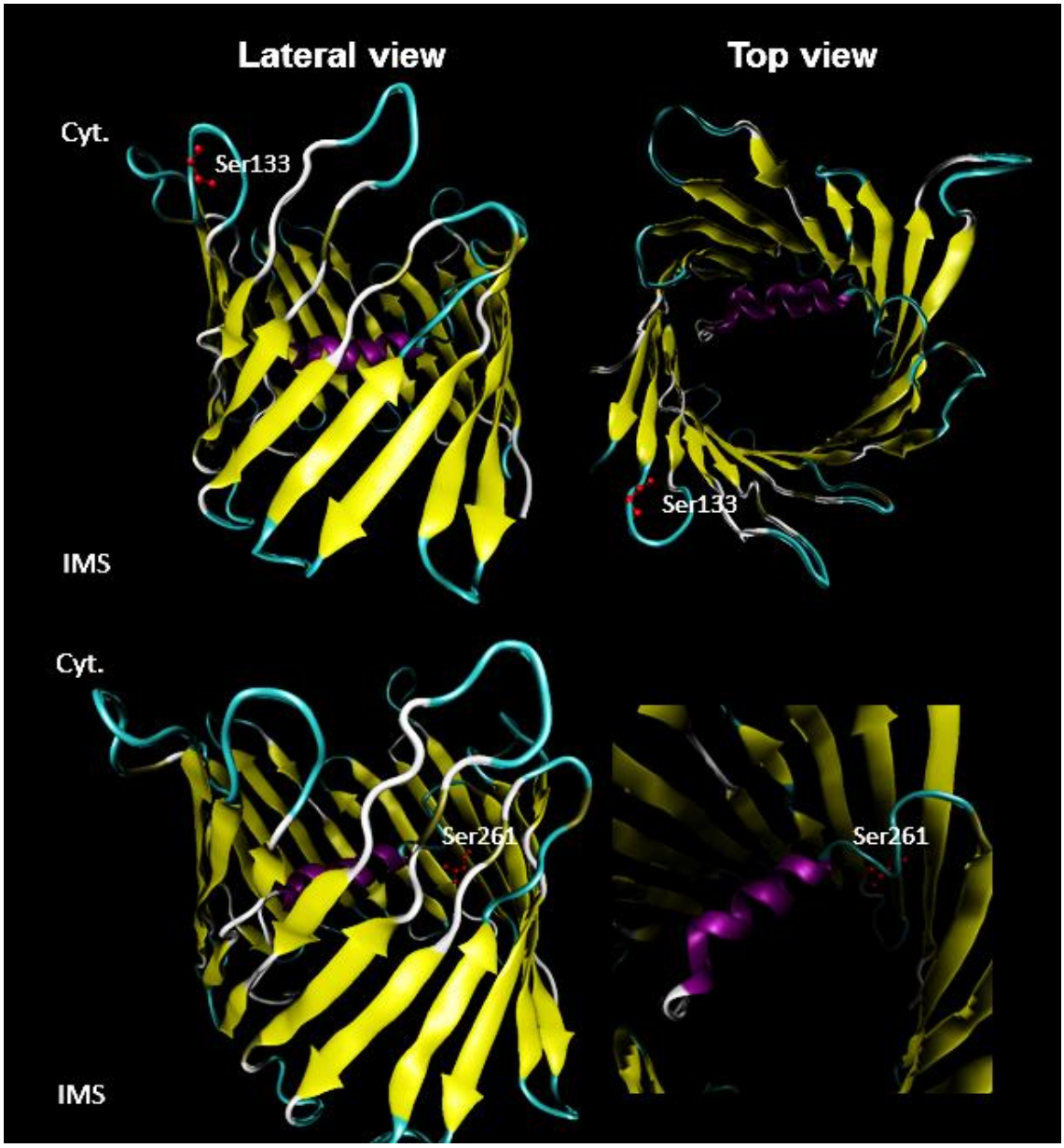

Figure 9 - Structure of human Por1p (2JK4) highlighting the phosphoresidues potential regulated by Sit4p (Bayrhuber, Meins et al. 2008). In this study it was considered the alignment of Por1p sequence from *S. cerevisiae* and human and the study of McDonald, Wydro et al. (2009) that predict the structure of Por1p in yeast. These two sequences have 24% of identify, 69 identical positions and 133 similar positions**.**

Table 4. Por1p phosphoresidues potential regulated by Sit4p. The secondary structure was assessed by mapping the residues onto the 3D x-ray crystallographic structure of human Por1p. The predicted kinases were assessed using a bioinformatic tool (Blom, Gammeltoft et al. 1999).

Sequence of the phosphorylated peptides	Phosphorylated amino acid	Secondary structure	Predicted Kinase
SPTFVGDLTMAHEGIVGGAEF GYDISAGSISR (133-164)	S133	Cyt. Loop	p38MAPK (HOG1) caM-II (CaM kinase) GSK3 (Rim11p) CDK5 (Pho85p)
QLLRPGVTLGVG**S**SFDAL (249-267)	S261	β-sheet	CKII

4.1.1 Por1p does not co-immunoprecipitates with Sit4p

In order to elucidate the mechanism by which Sit4p modulates the Por1p phosphorylation, we evaluated a possible direct interaction among these two proteins. A Sit4p-TAP construction was used for the immunoprecipitation assay (IP). After IP the possible interaction with Por1p was evaluated by SDS-PAGE and western blot. In spite of the high amount of Por1p in the input and the successful interaction between Sit4p-TAP and the beads, in the conditions used, it was not detected an interaction among Por1p and Sit4p (Figure 10).

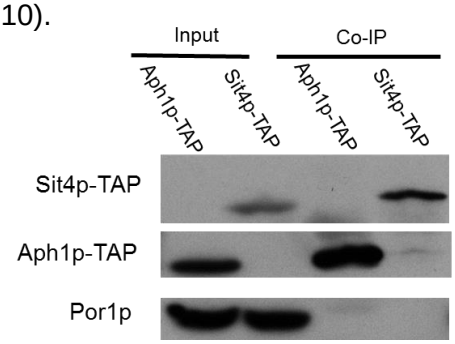

Figure 10 - Por1p does not co-immunoprecipitate with Sit4p-TAP. Cells expressing endogenously the Sit4p-TAP or Aph1-TAP fused proteins were grown to PDS phase and the proteins extracts were prepared. The fused proteins were immunoprecipitated, independently, using rabbit IgG−Agarose that recognize TAP. The presence of Por1p, Sit4p-TAP and Aph1p-TAP in the input and in the co-IP was examined by SDS-PAGE and western blot using an antibody that recognized the Por1p or the TAP. The Aph1-TAP was used as control in order to exclude possible nonspecific interactions between Por1p and the beads or with the TAP tag.

To evaluate the genetic interaction between *SIT4* and *QCR2* or *POR1*, double mutants were generated and characterized (see 4.2 and 4.3).

4.2. Role of Qcr2p on *sit4*Δ phenotypes

To assess if a genetic interaction occurs between *SIT4* and *QCR2*, which may support Qcr2p as being a Sit4p target, the *QCR2* gene was deleted in a *sit4*Δ strain. Several phenotypes were evaluated in this double mutant such as cellular growth, oxygen consumption, hydrogen peroxide resistance, chronological lifespan (CLS) and glycogen accumulation.

4.2.1. Analysis of cellular growth

Firstly, we assessed if *QCR2* deletion affects the growth of *sit4*Δ cells. Figure 11 shows that *sit4*Δ cells displayed a lower growth rate in the log phase but its cultures reached a higher cell density at stationary phase. The growth of *qcr2*Δ cells during log phase was similar to WT but stationary phase cultures of this mutant showed lower OD_{600} (13.8 and 12.0, respectively). Cellular growth in *sit4*Δ*qcr2*Δ double mutants was lower than expected based on either single mutation alone, indicating that there is a negative genetic interaction between *SIT4* and *QCR2* (synthetic sick).

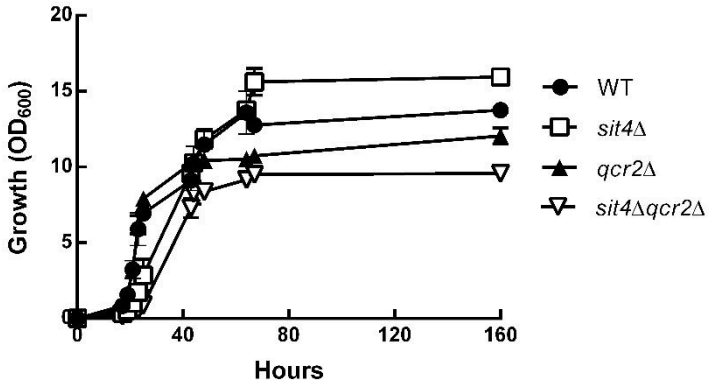

Figure 11 – Effect of *QCR2* deletion on cell growth. The yeast cells were grown in YPD medium. The growth was monitored by OD_{600} measurements over time. Values are obtained by three independent experiments.

4.2.2. *QCR2* deletion abolish the oxygen consumption of *sit4*Δ cells

The capacity to consume oxygen under glucose repressing conditions (at log phase of growth) is a distinct phenotype of *sit4*Δ cells (Bayrhuber, Meins et al. 2008, Barbosa, Osorio et al. 2011). In order to evaluate if this effect is dependent on Qcr2p and, consequently, of respiratory chain well work, the effect of *QCR2* deletion on the oxygen consumption rate (OCR) was monitored at log and PDS phase (Figure 12).

In Figure 12, it can be observed that the OCR was higher in *sit4*Δ cells when compared with WT at log phase (38.2 nmol mL^{-1} and 20.2 nmol mL^{-1}, respectively). At PDS phase, the OCR of *sit4*Δ was similar to WT cells (54.5 nmol mL^{-1} and 64.0 nmol mL^{-1}, respectively). In case of *qcr2*Δ cells, OCR was lower when compared to WT cells, being almost null in the PDS phase. The deletion of *QCR2* in *sit4*Δ mutants lead to a decrease of OCR to *qcr2*Δ levels.

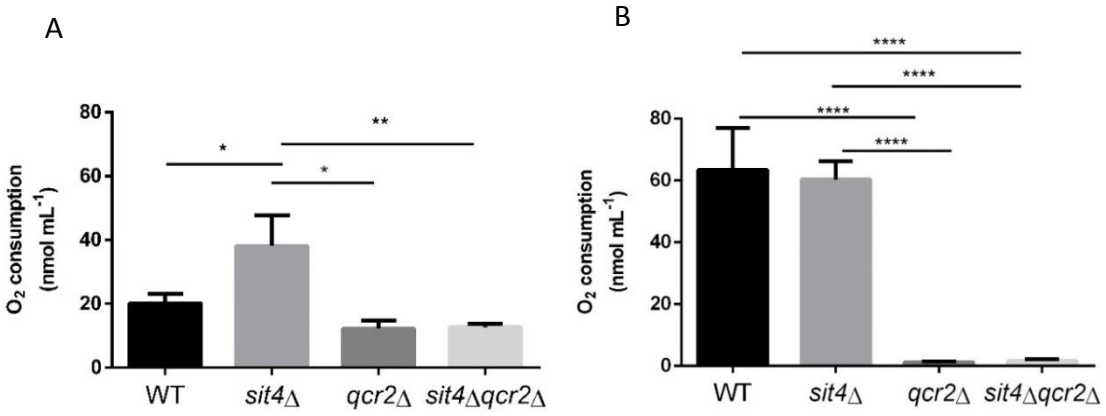

Figure 12 - **Role of Qcr2p in oxygen consumption of WT and *sit4*Δ cells.** The WT, *sit4*Δ, *por1*Δ and *sit4*Δ*por1*Δ cells were grown to A) log phase (OD_{600}=0.6) B) post-diauxic phase (OD_{600}=7-10) and the oxygen consumption rate was evaluated using a Clark oxygen electrode coupled to an Oxygraph system. Values were compared by one-way ANOVA *p< 0.05; **, p< 0.01; ***, p< 0.001; ****, p<0.0001 from at least three independent experiments.

4.2.3. The resistance to hydrogen peroxide, the extended longevity and the higher glycogen accumulation in *sit4*Δ cells are Qcr2p-dependent

Previous work showed that *sit4*Δ cells have a higher resistance to hydrogen peroxide (Barbosa, Osorio et al. 2011). To assess if this phenotype is dependent on Qcr2p, hydrogen peroxide resistance was evaluated in *sit4*Δ*qcr2*Δ double mutants. The cells were exposed to H_2O_2 (2.5 mM in the log phase; 250 mM in the PDS phase) during 30 min. The analysis of cell viability (Figure 13) shows that *sit4*Δ cells displayed a higher resistance to H_2O_2, while *qcr2*Δ mutant exhibited sensitivity to this oxidant. The sensitivity of *qcr*2Δ cells was moderate at log phase but significant at PDS (cell viability in WT and mutant cells was 55.7% and 6.8%, respectively). The *QCR2* deletion decreased hydrogen peroxide resistance of *sit4*Δ cells, being this effect more evident in PDS phase cells where the cell viability of *sit4*Δ mutant decreased from 93.7% to 52.4% (WT levels) (Figure 13).

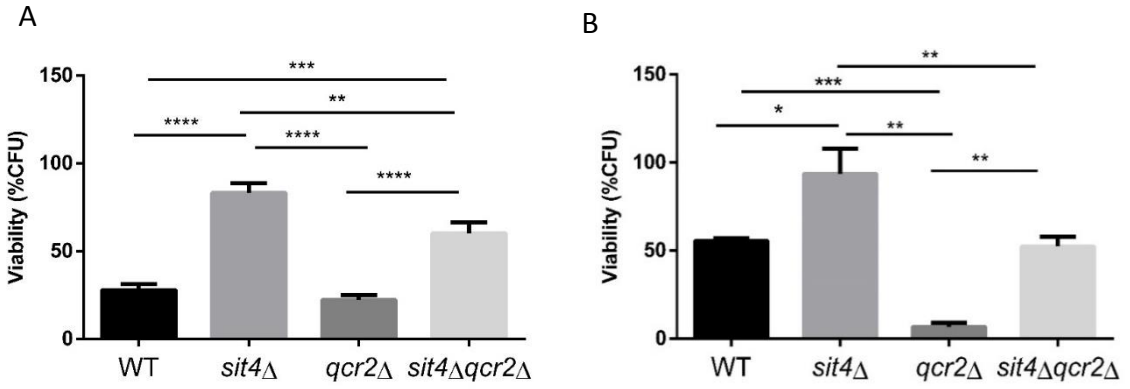

Figure 13 - **Role of Qcr2p in hydrogen peroxide resistance.** The WT, *sit4*Δ, *qcr2*Δ and *sit4*Δ*qcr2*Δ cells were grown to A) log phase (OD_{600}=0.6) or B) to post-diauxic phase (OD_{600}=7-10) in YPD medium and exposed for 30 min to 2.5 mM or 250 mM of H_2O_2, respectively. Cellular viability was expressed as the percentage of the colony-forming units (CFUs) (in relation to t0). Values were compared by one-way ANOVA *p< 0.05; **, p< 0.01; ***, p< 0.001; ****, p<0.0001 from at least three independent experiments.

Another particular characteristic of *sit4*Δ cells is the extended longevity. To investigate the role of Qcr2p in the extended longevity of *sit4*Δ cells, we assessed the chronological lifespan (CLS) of WT, *sit4*Δ, *qcr2*Δ and *sit4*Δ*qcr2*Δ cells. The mean lifespan of *qcr2*Δ cells was similar to WT cells (23.2 and 22 days, respectively) although *qcr2Δ* mutant lost viability faster during the initial stages of aging (e.g., at day 10 the viability was 40% lower in *qcr2Δ* mutants when comparing with WT) (Figure 14 and Table 5). Deletion of *QCR2* abolished the extended CLS of *sit4*Δ cells. The loss of viability in *sit4*Δ*qcr2*Δ cells was even faster to the observed in *qcr2*Δ cells and these double mutants showed a shorter mean lifespan (5 days) (Table 5).

Glycogen accumulation has being associated with high longevity of yeast cells (De Virgilio 2012). A previous study showed that *sit4*Δ cells have high levels of glycogen (Jablonka, Guzman et al. 2006). To determine if glycogen accumulation on *sit4*Δ cells is affected by *QCR2* deletion, we evaluated its levels by staining the colonies with iodine vapor. Our results show that glycogen accumulation in *sit4*Δ cells was suppressed by deletion of *QCR2* (Figure 14 B). Moreover, the levels of glycogen in *sit4*Δ*qcr2*Δ cells were similar to those observed in *qcr2*Δ single mutants and lower to that of WT cells.

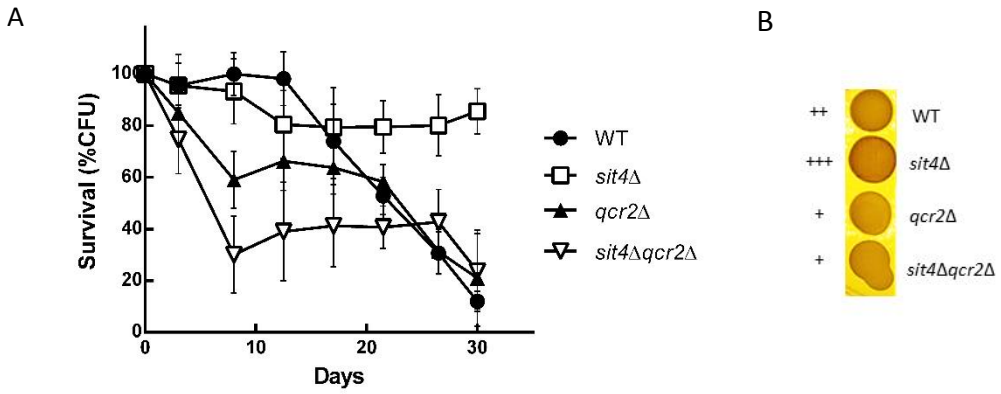

Figure 14 - The *QCR2* deletion decreases CLS and glycogen accumulation in *sit4*Δ cells. A) For chronological lifespan (CLS), cultures were grown and maintained in SC medium overtime. The cellular viability was expressed as the percentage of the colony-forming units (CFUs) (in relation to t0). Values are obtained by six independent experiments B) Glycogen content was evaluated qualitatively by exposition to iodine vapor of yeast colonies after 3 days of growth. A representative figure is shown out of 3 independent experiments. + low; ++ medium and +++ high **g**lycogen accumulation.

Table 5 - Mean lifespan of WT, *sit4*Δ, *qcr2*Δ and *sit4*Δ*qcr2*Δ cells. The mean lifespan was calculated as a time point when cultures reached 50% survival.

Strain	**Mean lifespan (days)**
WT	22
*sit4*Δ	Not reached
*qcr2*Δ	23.2
*sit4*Δqcr2Δ	5.5

4.3. Role of Por1p on *sit4*Δ phenotypes

To evaluate if Por1p plays a role in the Sit4p pathway, a mutant strain deleted for *POR1* in the *sit4*Δ background was studied.

4.3.1. Analysis of cellular growth

The analysis of cellular growth (Figure 15) shows that, comparing with WT, the *sit4*Δ and *por1*Δ cells displayed a delay in the entry to log phase but they reached a higher cell density in stationary phase (OD_{600} was 13.7 in WT, 15.9 in *sit4*Δ, and 15.1 in *por1*Δ cells). In *sit4*Δ*por1*Δ double mutants, cells showed a longer lag phase, entered the PDS phase 5 hours after the WT cells and reached a, lower OD_{600} (11.4) at stationary phase. These results suggest the existence of a negative genetic interaction between *SIT4* and *POR1* (synthetic sick) since cellular growth in *sit4*Δ*por1*Δ double mutants was lower than expected based on either single mutation alone.

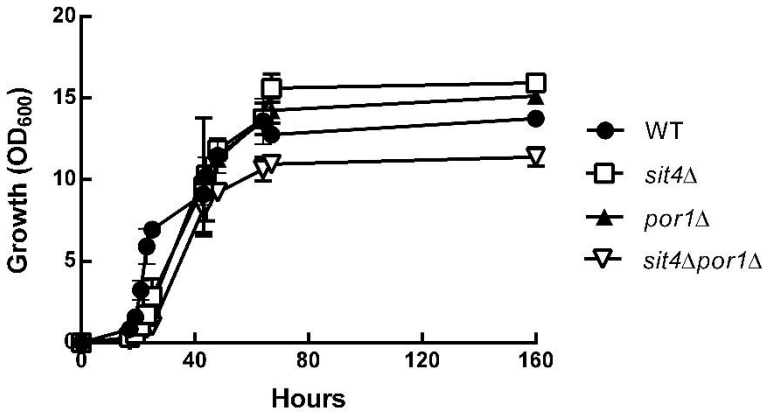

Figure 15 - Effect of *POR1* deletion on cell growth. The yeast cells were grown in YPD medium. The growth was monitored by OD_{600} measurements over time. Values are obtained by three independent experiments.

4.3.2. *POR1* deletion decreases the oxygen consumption rate of *sit4*Δ cells under respiratory conditions

In order to evaluate if the high OCR in *sit4*Δ cells is dependent on Por1p, the effect of *POR1* deletion was evaluated at log and PDS phase (Figure 16). As described before, at log phase, the oxygen consumption was higher in *sit4*Δ cells when compared with WT (33.9 nmol mL^{-1} and 13.9 nmol mL^{-1}, respectively). At PDS, the OCR of *sit4*Δ was similar to WT cells (54.5 nmol mL^{-1} and 64.0 nmol mL^{-1}, respectively). The deletion of *POR1* decreased the OCR of WT and *sit4*Δ cells in both phases. Notably, OCR increased 5.3-fold in *por1*Δ cells grown from the log (4.0 nmol mL^{-1}) to the PDS phase (21.5 nmol mL^{-1}). A similar increase (4.6-fold) was observed in WT cells (from 13.8 to 64.0 nmol mL^{-1}). This effect was not observed in *sit4*Δ*por1*Δ double mutants, which exhibited an OCR similar in both phases (at log phase 22.3 and PDS phase 28.6) and lower to that observed in *sit4*Δ cells (Figure 16).

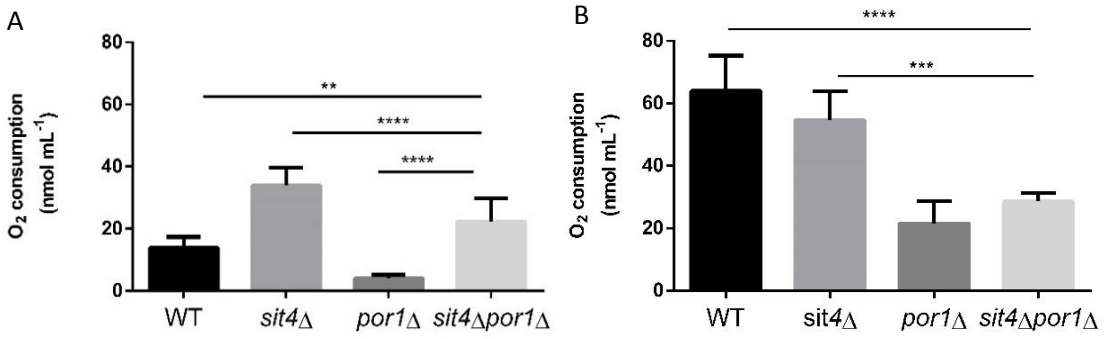

Figure 16 - Role of Por1p in oxygen consumption of WT and *sit4*Δ cells. The WT, *sit4*Δ, *por1*Δ and *sit4*Δ*por1*Δ were grown to A) log (OD_{600}=0.6) or B) PDS (OD_{600}=7-10) phase and the oxygen consumption was evaluated using a Clark oxygen electrode coupled to an Oxygraph system (Hansatech). Values were compared by one-way ANOVA *p< 0.05; **, p< 0.01; ***, p< 0.001; ****, p<0.0001 from at least three independent experiments.

4.3.3. Por1p deficiency impairs the mitochondrial localization of Isc1p but do not affect the mitochondrial network morphology

The mitochondrial localization of Isc1p, at late log phase, has been postulated as one of the events necessary for induction of the respiratory metabolism (Vaena de Avalos, Okamoto et al. 2004). Taking into account that the absence of Por1p decreases the OCR of *sit4*Δ cells, we evaluated whether this could be due to a decreased mitochondrial localization of Isc1p. For that, we monitored the localization of Isc1p by fluorescence microscopy, using cells expressing Isc1p-GFP (Figure 17 A).

We observed that the number of cells containing Isc1p-GFP in mitochondria increased in *sit4*Δ cells (61.8% vs 37.5% in WT) whereas it decreased in *por1*Δ cells (26.2%) (Figure 17 B). *POR1* deletion in *sit4*Δ cells decreased the % of cells with mitochondrial Isc1p-GFP to *por1*Δ levels. These results suggest that Por1p affects Isc1p mitochondrial localization. To assess whether Isc1p and Por1p could physically interact, we performed a co-immunoprecipitation assay. Under the conditions tested, an interaction between these proteins was not observed (Figure 17 C).

In absence of Isc1p, yeast cells show mitochondrial fragmentation (Teixeira, Medeiros et al. 2014). The decreased number of *por1*Δ cells exhibiting Isc1p in mitochondria led us to assess the mitochondrial network morphology by fluorescence microscopy. The results showed that *por1*Δ and sit4Δ*por1*Δ cells display a normal mitochondrial tubular network (chapter 8, Figure S2).

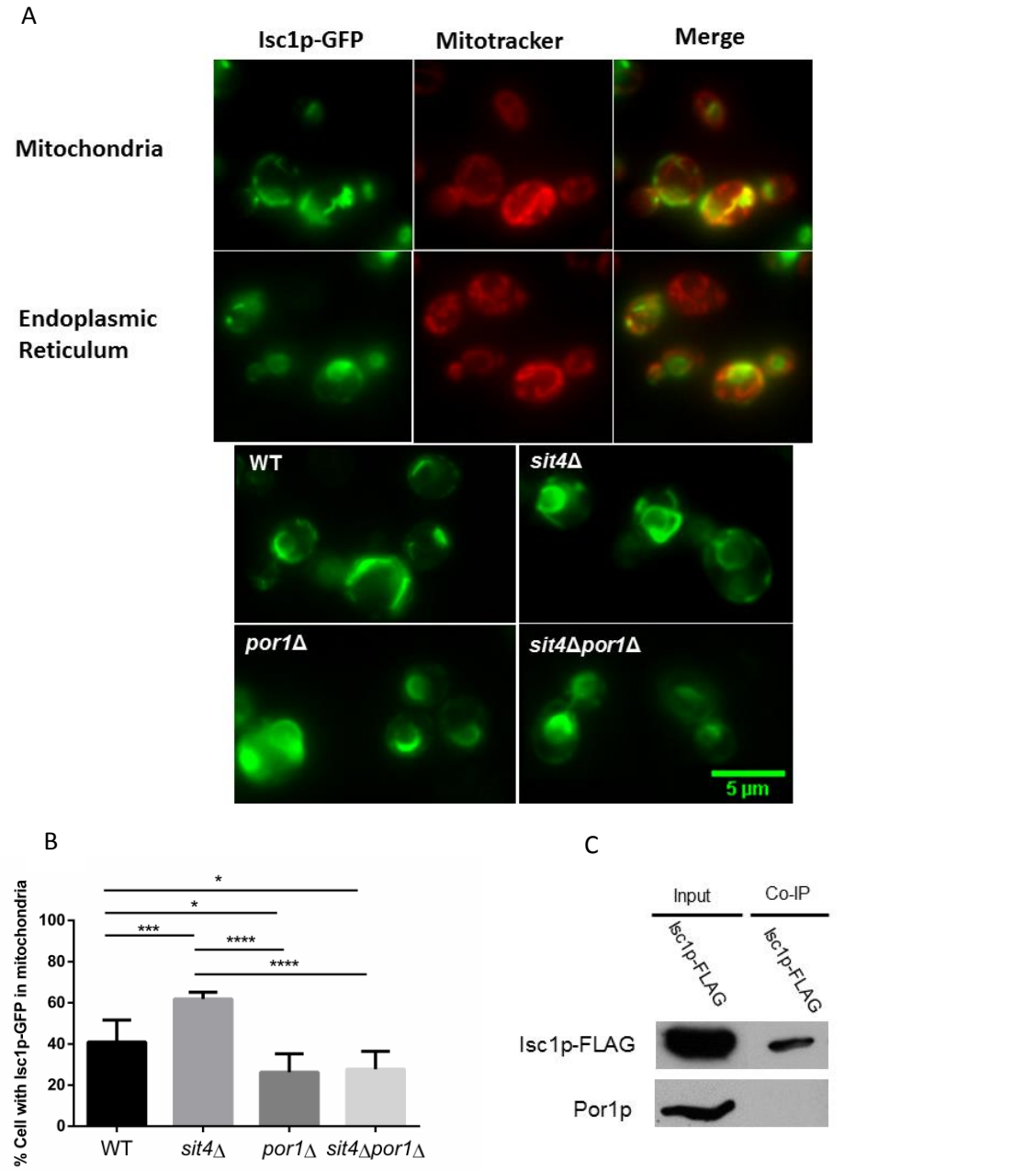

Figure 17 - Role of Por1p in mitochondrial localization of Isc1p. A) Intracellular localization of Isc1p-GFP (expressed from a GAL promoter) in WT, *sit4*Δ, *por1*Δ and *sit4*Δ*por1*Δ was examined by fluorescence microscopy. The counts were processed taking in account the typical morphology of endoplasmic reticulum and mitochondria. The MitoTracker Red CM-H_2XRos probe was used to stain the mitochondria. B) The % Isc1p-GFP in mitochondria was assessed using at least 300 cells of 3 different experiences. Values were compared by one-way ANOVA *p< 0.05; **, p< 0.01; ***, p< 0.001; ****, p<0.0001. C) Por1p does not co-immunoprecipitate with Isc1p-FLAG. Cells expressing Isc1p-FLAG fused protein were grown to OD_{600}=5 and the proteins extracts was prepared. The fused proteins were IP using an antibody that recognizes the FLAG. The presence of Por1p and Isc1p-FLAG in the input and in the co-IP was examined by SDS-PAGE and immunoblotting using antibodies for the respective proteins.

4.3.4. Sit4p plays a critical role in hydrogen peroxide sensitivity and CLS of *por1*Δ cells

Previous works showed an active role of Sit4p and Por1p on the modulation of H_2O_2 sensitivity (Pereira, Camougrand et al. 2007, Barbosa, Osorio et al. 2011). Thus in this present study was assessed the H_2O_2 sensitivity of the *sit4*Δ*por1*Δ mutants.

As before, *sit4*Δ cells showed resistance to H_2O_2 at both log and PDS phases. In contrast, *por1*Δ mutants were sensitive to H_2O_2 but only at the PDS phase. The oxidative stress resistance of *sit4*Δ*por1*Δ double mutants was similar to that observed in *sit4*Δ cells, indicating that the sensitivity of *por1*Δ was abolished by *SIT4* deletion. These results suggest a role of Sit4p in the H_2O_2 sensitivity of *por1*Δ cells at PDS phase (Figure 18).

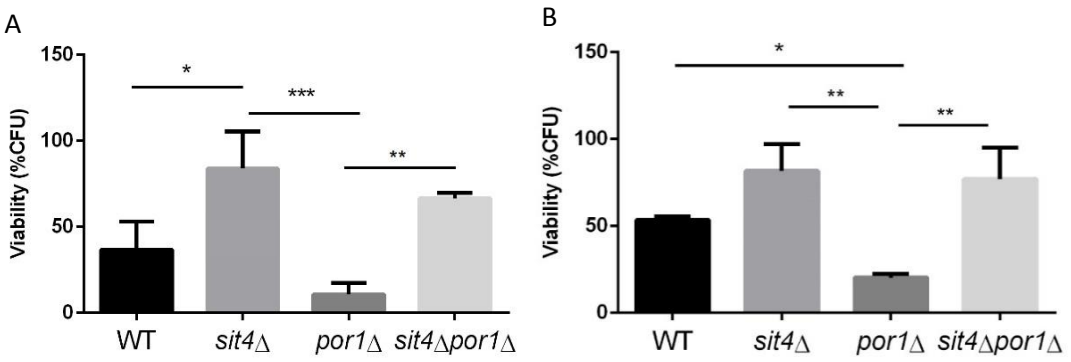

Figure 18 - Role of Por1p in hydrogen peroxide resistance of WT and *sit4*Δ cells. The WT, *sit4*Δ, *por1*Δ and *sit4*Δ*por1*Δ cells were grown to A) log phase (OD_{600}=0.6) or B) to post-diauxic phase (OD_{600}=7-10) in YPD medium and exposed for 30 min to 2.5 mM or 250 mM of H_2O_2, respectively. Cellular viability was expressed as the percentage of the colony-forming units (CFUs) (in relation to t0). Values were compared by one-way ANOVA *p< 0.05; **, p< 0.01; ***, p< 0.001; ****, p<0.0001 from at least three independent experiments.

As mentioned before, Sit4p plays an crucial role in aging (Barbosa, Osorio et al. 2011). To investigate the potential involvement of Por1p in *sit4*Δ longevity, we evaluated the CLS and medium lifespan of WT, *sit4*Δ, *por1*Δ and *sit4*Δ*por1*Δ cells (Figure 19 A). Mean lifespan was not affected in *por1*Δ cells (Table 6) but the initial loss of cellular viability occurred at a higher rate in this mutant comparing with WT cells. Notably, *SIT4* deletion increased the lifespan of *por1*Δ cells, with *sit4*Δ*por1*Δ double mutants remaining 100% viable up to day 21 of aging and a mean lifespan higher than days 30.

Due the association of glycogen accumulation with a higher longevity, the qualitatively evaluation of glycogen accumulation was performed. As described before, *sit4*Δ cells showed high levels of glycogen (Figure 19 B). In *por1*Δ cells, the glycogen content was similar to WT cells. However, *sit4*Δ*por1*Δ double mutants showed low levels of glycogen.

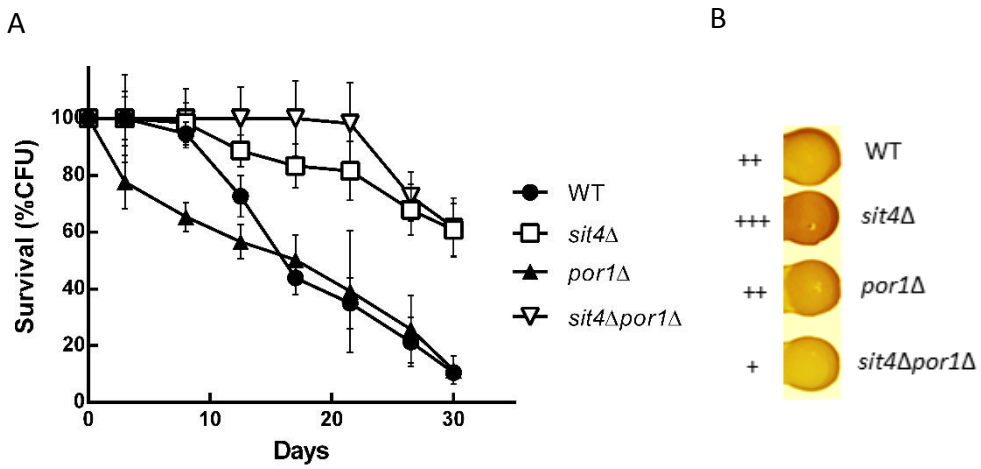

Figure 19 - The *sit4* deletion increases the CLS of *por1*Δ cells independently of glycogen accumulation. A) For chronological lifespan (CLS) cultures were grown and maintained in SC medium overtime. The cellular viability was expressed as the percentage of the colony-forming units (CFUs) (in relation to t0). Values are obtained by six independent experiments. B) Glycogen content was evaluated qualitatively by exposition to iodine vapor of yeast colonies after 3 days of growth. A representative figure is shown out of 3 independent experiments. +: low; ++: medium +++: high glycogen accumulation.

Table 6 - Mean lifespan of WT, *sit4*Δ, *por1*Δ and *sit4*Δ*por1*Δ. The mean lifespan was calculated as a time point when cultures reached 50% survival.

Strain	Mean lifespan (days)
WT	15.75
*sit4*Δ	Not reached
*por1*Δ	17
*sit4*Δ*por1*Δ	Not reached

4.3.5. Sit4p affects Sod1p activity in *por1*Δ cells

To investigate if *SIT4* deletion increased oxidative stress resistance and CLS of *por1*Δ cells through modulation of antioxidant defenses, we measured the activity of catalase and superoxide dismutase (Sod). As previously described in the literature, *sit4*Δ cells displayed a higher catalase activity comparatively to WT (9.8 U mg^{-1} and 7.1 U mg^{-1}, respectively) (Barbosa, Osorio et al. 2011) (Figure 20). However, the deletion of *SIT4* in *por1*Δ cells had no effect in catalase activity, suggesting that the increased resistance to H_2O_2 and the extended longevity of *por1*Δ*sit4*Δ cells are independent from this enzyme.

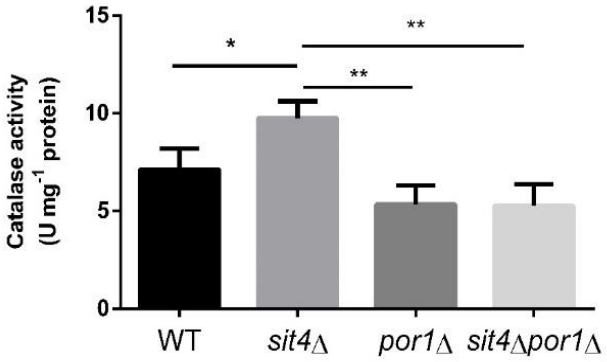

Figure 20 - Role of Sit4p in catalase activity of *por1*Δ cells. The catalase activity was analysed spectrophotometrically by following the decomposition of H_2O_2 at 240 nm. Protein extracts of WT, *sit4*Δ, *por1*Δ and *sit4*Δ*por1*Δ cells were obtained from cultures at post-diauxic phase (OD_{600}=7-10). Values were compared by one-way ANOVA *p< 0.05; **, p< 0.01; ***, p< 0.001; ****, p<0.0001 from at least three independent experiments.

Regarding Sods, the WT, *sit4*Δ and *por1*Δ cells showed similar activities of Sod1p and Sod2p. Notably, *por1*Δ*sit4*Δ double mutants showed an over activation of Sod1p to levels 5.8-fold and 2.6-fold higher to those observed in WT and *por1*Δ cells, respectively (Figure 21).

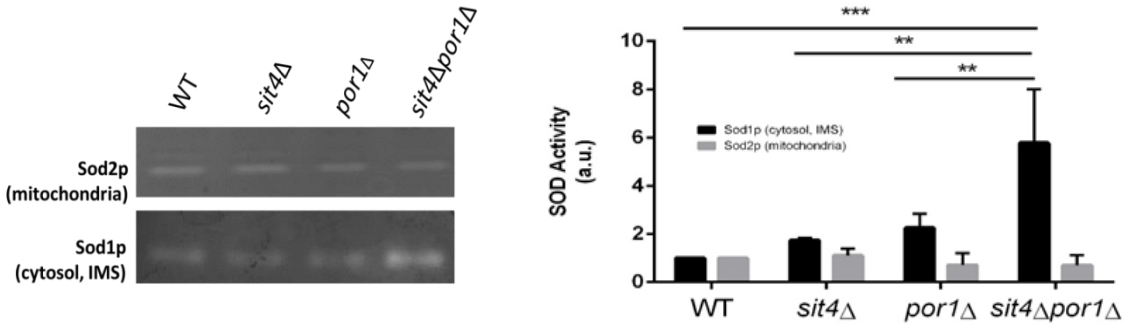

Figure 21 - Role of Sit4p in Sodp activity of *por1*Δ cells. The Sod activity was determined *in situ* after separation of proteins by native polyacrylamide gel electrophoresis. Protein extracts of WT, *sit4*Δ, *por1*Δ and *sit4*Δ*por1*Δ cells were obtained from cultures at post-diauxic phase (OD_{600}=7-10). The analysis of Sod activity was based on the inhibition of NBT reduction by superoxide radicals. Values were compared by one-way ANOVA *p< 0.05; **, p< 0.01; ***, p< 0.001; ****, p<0.0001 from four independent experiments.

CHAPTER 5
DISCUSSION

Sit4p is a Ser/Thr phosphatase associated with two well-known signalling pathways, being inhibited by TORC1 and activated by ceramide (Bastians and Ponstingl 1996, Di Como and Arndt 1996). This protein plays a crucial role in mitochondrial function and redox homeostasis, impacting longevity (Jablonka, Guzman et al. 2006, Barbosa, Osorio et al. 2011). The involvement of Sit4p with mitochondria was recently reinforced by a study from Garipler, Mutlu et al. (2014), in which the authors demonstrate that *SIT4* deletion suppresses phenotypes of rho0 cells (cells lacking mitochondrial genome) such as reduced proliferation, mitochondrial protein import defects, lower mitochondrial membrane potential and nuclear transcriptional response associated with mtDNA damage. However the mechanism(s) involved in these processe(s) remain to be elucidated.

Due to the involvement of Sit4p in mitochondrial function, a proteomic study was performed in our group to identify potential mitochondrial targets. In the absence of *SIT4*, nine proteins were found to be hyperphosphorylated (Pereira C. et al. unpublished data). Taking into account that Sit4p is a phosphatase, and phosphorylation is the most prevalent post-transduction alteration that modulates protein function, changes in mitochondrial phosphorylation pattern may be associated with *sit4*Δ mutant phenotypes. In this work, two of the identified potential Sit4p targets, Qcr2p and Por1p were studied.

Qcr2p/Cor2p is the subunit II of the mitochondrial complex III and it has a high homology to the Zn-binding matrix processing peptidase (Braun and Schmitz 1995, Smith, Fox et al. 2012). In yeast, this protein does not have proteolytic activity due to an incomplete active site, but has been suggested to play an important role in protecting cytochrome b from degradation (Braun, Emmermann et al. 1992, Zara, Conte et al. 2007). Most importantly, Qcr2p was shown to be essential for complex III assembly (Zara, Conte et al. 2007).

The role of Qcr2p phosphorylation is poorly characterized. The study of Ohlmeier, Hiltunen et al. (2010) showed that Qcr2p phosphorylation increases during the switch from log to PDS phase. This fact leads us to speculate that Qcr2p phosphorylation improves its function since mitochondrial activity increases at the PDS phase. However, the modulation of protein function by phosphorylation is very complex and its effects may depend on the residue that is phosphorylated. For this reason more studies will be necessary to characterize the exact function of Qcr2p phosphorylation in *sit4*Δ phenotypes.

In our genetic studies, it was observed a negative interaction between *QCR2* and *SIT4*, since the deletion of *QCR2* reverted most of *sit4*Δ mutant phenotypes. Our results are in agreement with previous studies since the activity of the respiratory chain, which

is impaired in the absence of Qcr2p, has been associated with a high hydrogen peroxide resistance and extended longevity (Bonawitz, Chatenay-Lapointe et al. 2007, Aerts, Zabrocki et al. 2009, Ocampo, Liu et al. 2012). This effect has been attributed to the $O_2^{\bullet-}$ produced by the respiratory chain (namely by complex III) during stress or in an early stage of growth that induces antioxidant defenses, promoting resistance and extended lifespan (Pan 2011, Thorpe, Reodica et al. 2013). In addition, the lower glycogen content observed in *sit4*Δ*qcr2*Δ cells may also contributes to its shortened lifespan, since glycogen is important for cellular longevity (De Virgilio 2012). In respiratory deficient cells, as is the case of *sit4*Δ*qcr2*Δ mutants, the lower levels of glycogen are attributed to its utilization as fuel and/or the inhibition of glycogen synthase (Gsy2p) (Yang, Chun et al. 1998). However, it is important to emphasize that our results indicate which respiration/glycogen content seem to influence the CLS only at the initial stages of aging since in *qcr2*Δ and *sit4*Δ*qcr2*Δ cells, the maximum CLS was similar to WT cells. This indicates that the *qcr2*Δ and *sit4*Δ*qcr2*Δ cells are able to adapt through a mechanism that remains to be clarified.

Taken together, these results suggest that Qcr2p may be a downstream target of Sit4p. Sit4p mediated dephosphorylation of Qcr2p may decrease complex III activity affecting both stress resistance and CLS. Future work, using non-phosphorylatable mutants of Qcr2p in the *sit4*Δ background will be important to test this hypothesis.

Por1p is another mitochondrial protein hyperphophorylated in cells lacking Sit4p. This protein is an anion selective channel dependent of voltage that allows the passage of metabolites between the intermembrane space and cytosol (Lee, Xu et al. 1998). This channel display two states: 1) the open state, that in mammals is associated with low mitochondrial membrane potential (<30 mV) and allows the passage of anions (such as ATP, succinate, citrate and phosphate); 2) the closed state, associated with a high mitochondrial membrane potential (>30 mV) and an exchange in channel selectivity allowing the passage of cations (Colombini 1989, Colombini, Blachly-Dyson et al. 1996, Rostovtseva and Colombini 1997). Beyond this function Por1p plays a critical role in the modulation of yeast redox homeostasis, stress resistance and maintenance of mitochondrial osmotic stability (Sanchez, Pearce et al. 2001, Pereira, Camougrand et al. 2007, Galganska, Karachitos et al. 2010, Galganska, Karachitos et al. 2010). The role of Por1p phosphorylation in yeast is unknown, though in mammalians, depending on the phosphorylated residue, it is important to prevent the interaction with Hexokinase-2, to promote the interaction with tubulin (decreasing the respiration), to keep the mitochondrial membrane potential or to favor the closed state of the channel (Chen,

Gaczynska et al. 2010, Herrera, Diaz et al. 2011, Sheldon, Maldonado et al. 2011, Kerner, Lee et al. 2012).

In this study we found two novel Por1p phosphorylated residues (Ser133 and Ser261) that are potentially regulated by Sit4p. The *in silico* analysis suggest that Ser133 (localized in cytosolic loop) may be more accessible to the action of kinases/phosphatases than Ser261 (localized in β-sheet). However, human Por1p can be phosphorylated in Ser193 (Chen, Gaczynska et al. 2010), which has a position similar to that of yeast Por1p-Ser261 indicating phosphorylation in this region is possible. The regulation of Por1p phosphorylation by Sit4p does not seem to be direct since our results suggest that these proteins do not interact. This fact leads us to propose an alternative mechanism which may involve the Snf1p kinase [in our networKIN analysis the Snf1p or AMPK (human homologue) is not covered]. Sit4p has a direct effect on Snf1p phosphorylation leading to its inhibition (Cherkasova, Qiu et al. 2010). In addition, a recent study showed a direct interaction between Snf1p and Por1p (Strogolova, Orlova et al. 2012). We postulate that Snf1p is activated in cells lacking Sit4p, leading to Por1p hyperphophorylation. Future studies will be necessary to ascertain this possibility.

In the genetic analysis we found a negative interaction between *POR1* and *SIT4* namely on cellular growth and oxygen consumption rate (OCR). This negative interaction may be correlated with the function of the cannel. Sit4p can modulate the activity of Por1p affecting respiration. However, Por1p is only partially necessary to mitochondrial respiration since Por1p deficient cells are able to grow on respiratory sources (Dihanich, Suda et al. 1987, Blachly-Dyson, Song et al. 1997) and we observed a basal OCR in *por1*Δ cells at PDS.

Alternatively, the genetic interaction may be related with Isc1p, a protein that functions upstream of Sit4p (Barbosa, Osorio et al. 2011). The absence of Por1p impairs the mitochondrial localization of Isc1p (inositol phosphosphingolipid phospholipase C) in WT and *sit4*Δ cells which could contribute to the reduced OCR of *por1*Δ and *sit4*Δ*por1*Δ cells. Indeed, Isc1p is localized in mitochondria on respiring cells and its deficiency impairs the induction of genes required for mitochondrial respiration (Vaena de Avalos, Okamoto et al. 2004, Kitagaki, Cowart et al. 2009). However a recent study does not support a strict requirement of Isc1p in mitochondria for OCR, since *sch9*Δ mutants exhibit a high OCR despite decreased levels of Isc1p in mitochondria (Swinnen, Wilms et al. 2014). Despite the absence of Por1p impairs the mitochondrial localization of Isc1p, our results indicate there was no physical interaction between these proteins, which may exclude the hypothesis that Por1p could help anchor Isc1p to mitochondria. Interestingly

a genetic association has already been reported for these two genes (*POR1* and *ISC1*) (Hoppins, Collins et al. 2011).

Additionally, our results showed that deletion of *SIT4* increased hydrogen peroxide resistance and lifespan of *por1*Δ cells which is associated with a strong increase in Sod1p activity. The Sod1p was suggested as important to stress resistance and longevity of yeast cells since *SOD1* deletion and overexpression were associated with a shorter and extended longevity respectively, coupled to an increased resistance to endogenous stress, in the latter situation (Longo, Gralla et al. 1996, Fabrizio and Longo 2003, Harris, Bachler et al. 2005).

CHAPTER 6
CONCLUSIONS AND FUTURE PROSPECTS

In summary, we observed an increase in the phosphorylation of Qcr2p and Por1p in cells lacking Sit4p and genetic interactions between *SIT4* and *QCR2* or *POR1*. Our data show that *sit4*Δ phenotypes such as the higher oxygen consumption rate, resistance to hydrogen peroxide, increased chronological lifespan and glycogen accumulation are Qcr2p-dependent. To assess whether this effect is specific for Qcr2p or if it is a general effect of disturbing the respiratory chain, the genetic interaction of *SIT4* with another gene that codify for respiratory chain proteins should be performed.

Our results also suggest a role of Por1p in the improvement of mitochondrial respiration in *sit4*Δ cells. However, the deletion of *SIT4* still increased hydrogen peroxide resistance and lifespan of *por1*Δ cells, suggesting that Por1p contributes to a subset of *sit4*Δ phenotypes. Notably, *sit4*Δ*por1*Δ double mutants exhibited high levels of Sod1p activity. To evaluate if the induction of this antioxidant defense contributes to hydrogen peroxide resistance and to the longevity of *sit4*Δ*por1*Δ cells, the effect of *SOD1* deletion on these phenotypes must be characterized.

Taken together, these results offer new insights on Sit4p signaling pathways pointing to a possible involvement of Por1p and Qcr2p regulation by phosphorylation. The challenge for future studies is to put these modifications in a physiological perspective. The mutation of putative phosphorylated residues into Ala (non-phosphomimetic) and Glu (phosphomimetic) residues is required to uncover the function of Por1p and Qcr2p phosphorylation in *sit4*Δ phenotypes. The role of phosphorylation in those residues in the function of Por1p or Qcr2p should also be evaluated since nothing is known regarding the regulation of these proteins by phosphorylation.

The mechanism(s) that promotes Por1p and Qcr2p phosphorylation in Sit4p deficient cells remains to be characterized. Our results did not show a physical interaction between Sit4p and Por1p, raising the hypothesis that at least Por1p may not be directly regulated by Sit4p. Since Sit4p is known to inhibit the Snf1p kinase, the increase in Por1p and Qcr2p phosphorylation may be associated with Snf1p activation, a hypothesis to be tested in futures studies.

CHAPTER 7
REFERENCES

Adam-Vizi, V. and C. Chinopoulos (2006). "Bioenergetics and the formation of mitochondrial reactive oxygen species." Trends Pharmacol Sci **27**(12): 639-645.

Aebi, H. (1984). "Catalase in vitro." Methods Enzymol **105**: 121-126.

Aerts, A. M., P. Zabrocki, I. E. Francois, D. Carmona-Gutierrez, G. Govaert, C. Mao, B. Smets, F. Madeo, J. Winderickx, B. P. Cammue and K. Thevissen (2008). "Ydc1p ceramidase triggers organelle fragmentation, apoptosis and accelerated ageing in yeast." Cell Mol Life Sci **65**(12): 1933-1942.

Aerts, A. M., P. Zabrocki, G. Govaert, J. Mathys, D. Carmona-Gutierrez, F. Madeo, J. Winderickx, B. P. A. Cammue and K. Thevissen (2009). "Mitochondrial dysfunction leads to reduced chronological lifespan and increased apoptosis in yeast." FEBS Letters **583**(1): 113-117.

Aggarwal, M. and R. M. Brosh, Jr. (2010). "Genetic studies of human DNA repair proteins using yeast as a model system." J Vis Exp(37): 1-5.

Almeida, T., M. Marques, D. Mojzita, M. A. Amorim, R. D. Silva, B. Almeida, P. Rodrigues, P. Ludovico, S. Hohmann, P. Moradas-Ferreira, M. Corte-Real and V. Costa (2008). "Isc1p plays a key role in hydrogen peroxide resistance and chronological lifespan through modulation of iron levels and apoptosis." Mol Biol Cell **19**(3): 865-876.

Angeles de la Torre-Ruiz, M., J. Torres, J. Arino and E. Herrero (2002). "Sit4 is required for proper modulation of the biological functions mediated by Pkc1 and the cell integrity pathway in *Saccharomyces cerevisiae*." J Biol Chem **277**(36): 33468-33476.

Arndt, K. T., C. A. Styles and G. R. Fink (1989). "A suppressor of a HIS4 transcriptional defect encodes a protein with homology to the catalytic subunit of protein phosphatases." Cell **56**(4): 527-537.

Attardi, G. and G. Schatz (1988). "Biogenesis of mitochondria." Annu Rev Cell Biol **4**: 289-333.

Barbosa, A. D., J. Graca, V. Mendes, S. R. Chaves, M. A. Amorim, M. V. Mendes, P. Moradas-Ferreira, M. Corte-Real and V. Costa (2012). "Activation of the Hog1p kinase in Isc1p-deficient yeast cells is associated with mitochondrial dysfunction, oxidative stress sensitivity and premature aging." Mech Ageing Dev **133**(5): 317-330.

Barbosa, A. D., H. Osorio, K. J. Sims, T. Almeida, M. Alves, J. Bielawski, M. A. Amorim, P. Moradas-Ferreira, Y. A. Hannun and V. Costa (2011). "Role for Sit4p-dependent mitochondrial dysfunction in mediating the shortened chronological lifespan and oxidative stress sensitivity of Isc1p-deficient cells." Mol Microbiol **81**(2): 515-527.

Bastians, H. and H. Ponstingl (1996). "The novel human protein serine/threonine phosphatase 6 is a functional homologue of budding yeast Sit4p and fission yeast ppe1, which are involved in cell cycle regulation." J Cell Sci **109 (Pt 12)**: 2865-2874.

Bayrhuber, M., T. Meins, M. Habeck, S. Becker, K. Giller, S. Villinger, C. Vonrhein, C. Griesinger, M. Zweckstetter and K. Zeth (2008). "Structure of the human voltage-dependent anion channel." Proc Natl Acad Sci U S A **105**(40): 15370-15375.

Bell, E. L., T. A. Klimova, J. Eisenbart, P. T. Schumacker and N. S. Chandel (2007). "Mitochondrial reactive oxygen species trigger hypoxia-inducible factor-dependent extension of the replicative life span during hypoxia." Mol Cell Biol **27**(16): 5737-5745.

Bhandari, D., J. Zhang, S. Menon, C. Lord, S. Chen, J. R. Helm, K. Thorsen, K. D. Corbett, J. C. Hay and S. Ferro-Novick (2013). "Sit4p/PP6 regulates ER-to-Golgi traffic by controlling the dephosphorylation of COPII coat subunits." Mol Biol Cell **24**(17): 2727-2738.

Blachly-Dyson, E., J. Song, W. J. Wolfgang, M. Colombini and M. Forte (1997). "Multicopy suppressors of phenotypes resulting from the absence of yeast VDAC encode a VDAC-like protein." Mol Cell Biol **17**(10): 5727-5738.

Blom, N., S. Gammeltoft and S. Brunak (1999). "Sequence and structure-based prediction of eukaryotic protein phosphorylation sites." J Mol Biol **294**(5): 1351-1362.

Bonawitz, N. D., M. Chatenay-Lapointe, Y. Pan and G. S. Shadel (2007). "Reduced TOR signaling extends chronological life span via increased respiration and upregulation of mitochondrial gene expression." Cell Metab **5**(4): 265-277.

Bratic, A., N.-G. Larsson, xF and ran (2013). "The role of mitochondria in aging." The Journal of Clinical Investigation **123**(3): 951-957.

Braun, H. P., M. Emmermann, V. Kruft and U. K. Schmitz (1992). "The general mitochondrial processing peptidase from potato is an integral part of cytochrome c reductase of the respiratory chain." Embo j **11**(9): 3219-3227.

Braun, H. P. and U. K. Schmitz (1995). "Are the 'core' proteins of the mitochondrial bc1 complex evolutionary relics of a processing protease?" Trends Biochem Sci **20**(5): 171-175.

Brunelle, J. K., E. L. Bell, N. M. Quesada, K. Vercauteren, V. Tiranti, M. Zeviani, R. C. Scarpulla and N. S. Chandel (2005). "Oxygen sensing requires mitochondrial ROS but not oxidative phosphorylation." Cell Metab **1**(6): 409-414.

Butler, J., G. G. Jayson and A. J. Swallow (1975). "The reaction between the superoxide anion radical and cytochrome c." Biochim Biophys Acta **408**(3): 215-222.

Cameroni, E., N. Hulo, J. Roosen, J. Winderickx and C. De Virgilio (2004). "The novel yeast PAS kinase Rim 15 orchestrates G0-associated antioxidant defense mechanisms." Cell Cycle **3**(4): 462-468.

Carlson, M. (1999). "Glucose repression in yeast." Curr Opin Microbiol **2**(2): 202-207.

Chance, B., H. Sies and A. Boveris (1979). "Hydroperoxide metabolism in mammalian organs." Physiol Rev **59**(3): 527-605.

Chaudhari, P., Z. Ye and Y. Y. Jang (2014). "Roles of reactive oxygen species in the fate of stem cells." Antioxid Redox Signal **20**(12): 1881-1890.

Chen, K., M. T. Kirber, H. Xiao, Y. Yang and J. F. Keaney, Jr. (2008). "Regulation of ROS signal transduction by NADPH oxidase 4 localization." J Cell Biol **181**(7): 1129-1139.

Chen, Y., M. Gaczynska, P. Osmulski, R. Polci and D. J. Riley (2010). "Phosphorylation by Nek1 regulates opening and closing of voltage dependent anion channel 1." Biochem Biophys Res Commun **394**(3): 798-803.

Cherkasova, V., H. Qiu and A. G. Hinnebusch (2010). "Snf1 promotes phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 by activating Gcn2 and inhibiting phosphatases Glc7 and Sit4." Mol Cell Biol **30**(12): 2862-2873.

Cherkasova, V. A. and A. G. Hinnebusch (2003). "Translational control by TOR and TAP42 through dephosphorylation of eIF2α kinase GCN2." Genes & Development **17**(7): 859-872.

Chow, C. K., W. Ibrahim, Z. Wei and A. C. Chan (1999). "Vitamin E regulates mitochondrial hydrogen peroxide generation." Free Radic Biol Med **27**(5-6): 580-587.

Circu, M. L. and T. Y. Aw (2010). "Reactive oxygen species, cellular redox systems, and apoptosis." Free Radic Biol Med **48**(6): 749-762.

Clayton, D. A. (1991). "Replication and transcription of vertebrate mitochondrial DNA." Annu Rev Cell Biol **7**: 453-478.

Colombini, M. (1989). "Voltage gating in the mitochondrial channel, VDAC." T J Membr Biol **111**(2): 103-111.

Colombini, M., E. Blachly-Dyson and M. Forte (1996). VDAC, a Channel in the Outer Mitochondrial Membrane. Ion Channels. T. Narahashi, Springer US. **4:** 169-202.

Conconi, A. (2008). Yeast as a Model System to Study DNA Damage and DNA Repair. Sourcebook of Models for Biomedical Research. P. M. Conn, Humana Press**:** 445-453.

de Assis, L. J., R. B. Zingali, C. A. Masuda, S. P. Rodrigues and M. Montero-Lomeli (2013). "Pyruvate decarboxylase activity is regulated by the Ser/Thr protein phosphatase Sit4p in the yeast *Saccharomyces cerevisiae*." FEMS Yeast Res **13**(6): 518-528.

De Virgilio, C. (2012). "The essence of yeast quiescence." FEMS Microbiol Rev **36**(2): 306-339.

del Rio, L. A., L. M. Sandalio, J. M. Palma, P. Bueno and F. J. Corpas (1992). "Metabolism of oxygen radicals in peroxisomes and cellular implications." Free Radic Biol Med **13**(5): 557-580.

Denoth Lippuner, A., T. Julou and Y. Barral (2014). "Budding yeast as a model organism to study the effects of age." FEMS Microbiol Rev **38**(2): 300-325.

Di Como, C. J. and K. T. Arndt (1996). "Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases." Genes Dev **10**(15): 1904-1916.

Dickinson, B. C. and C. J. Chang (2011). "Chemistry and biology of reactive oxygen species in signaling or stress responses." Nat Chem Biol **7**(8): 504-511.

Dickson, R. C. (2008). "Thematic review series: sphingolipids. New insights into sphingolipid metabolism and function in budding yeast." J Lipid Res **49**(5): 909-921.

Dihanich, M., K. Suda and G. Schatz (1987). "A yeast mutant lacking mitochondrial porin is respiratory-deficient, but can recover respiration with simultaneous accumulation of an 86-kd extramitochondrial protein." Embo j **6**(3): 723-728.

Duchen, M. R. (2000). "Mitochondria and calcium: from cell signalling to cell death." J Physiol **529 Pt 1**: 57-68.

Duvel, K., A. Santhanam, S. Garrett, L. Schneper and J. R. Broach (2003). "Multiple roles of Tap42 in mediating rapamycin-induced transcriptional changes in yeast." Mol Cell **11**(6): 1467-1478.

Eisenberg, T. and S. Buttner (2013). "Lipids and cell death in yeast." FEMS Yeast Res.

Eleutherio, E. C., P. S. Araujo and A. D. Panek (1993). "Protective role of trehalose during heat stress in *Saccharomyces cerevisiae*." Cryobiology **30**(6): 591-596.

Epstein, S., G. A. Castillon, Y. Qin and H. Riezman (2012). "An essential function of sphingolipids in yeast cell division." Mol Microbiol **84**(6): 1018-1032.

Fabrizio, P., L. L. Liou, V. N. Moy, A. Diaspro, J. S. Valentine, E. B. Gralla and V. D. Longo (2003). "SOD2 functions downstream of Sch9 to extend longevity in yeast." Genetics **163**(1): 35-46.

Fabrizio, P. and V. D. Longo (2003). "The chronological life span of *Saccharomyces cerevisiae*." Aging Cell **2**(2): 73-81.

Fabrizio, P., F. Pozza, S. D. Pletcher, C. M. Gendron and V. D. Longo (2001). "Regulation of longevity and stress resistance by Sch9 in yeast." Science **292**(5515): 288-290.

Fernandez-Sarabia, M. J., A. Sutton, T. Zhong and K. T. Arndt (1992). "SIT4 protein phosphatase is required for the normal accumulation of SWI4, CLN1, CLN2, and HCS26 RNAs during late G1." Genes Dev **6**(12a): 2417-2428.

Flohe, L. and F. Otting (1984). "Superoxide dismutase assays." Methods Enzymol **105**: 93-104.

Fontana, L., L. Partridge and V. D. Longo (2010). "Extending healthy life span--from yeast to humans." Science **328**(5976): 321-326.

Forman, H. J., M. Maiorino and F. Ursini (2010). "Signaling functions of reactive oxygen species." Biochemistry **49**(5): 835-842.

Francois, J. and J. L. Parrou (2001). "Reserve carbohydrates metabolism in the yeast *Saccharomyces cerevisiae*." FEMS Microbiol Rev **25**(1): 125-145.

Fridovich, I. (1995). "Superoxide radical and superoxide dismutases." Annu Rev Biochem **64**: 97-112.

Galganska, H., A. Karachitos, M. Baranek, M. Budzinska, J. Jordan and H. Kmita (2010). "Viability of Saccharomyces cerevisiae cells following exposure to H_2O_2 and protective effect of minocycline depend on the presence of VDAC." Eur J Pharmacol **643**(1): 42-47.

Galganska, H., A. Karachitos, M. Wojtkowska, O. Stobienia, M. Budzinska and H. Kmita (2010). "Communication between mitochondria and nucleus: putative role for VDAC in reduction/oxidation mechanism." Biochim Biophys Acta **1797**(6-7): 1276-1280.

Garipler, G., N. Mutlu, N. A. Lack and C. D. Dunn (2014). "Deletion of conserved protein phosphatases reverses defects associated with mitochondrial DNA damage in *Saccharomyces cerevisiae*." Proc Natl Acad Sci U S A **111**: 1473-1478

Garreau, H., R. N. Hasan, G. Renault, F. Estruch, E. Boy-Marcotte and M. Jacquet (2000). "Hyperphosphorylation of Msn2p and Msn4p in response to heat shock and the diauxic shift is inhibited by cAMP in *Saccharomyces cerevisiae*." Microbiology **146 (Pt 9)**: 2113-2120.

Gasch, A. P., P. T. Spellman, C. M. Kao, O. Carmel-Harel, M. B. Eisen, G. Storz, D. Botstein and P. O. Brown (2000). "Genomic expression programs in the response of yeast cells to environmental changes." Mol Biol Cell **11**(12): 4241-4257.

Gietz, R. D. and R. H. Schiestl (2007). "High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method." Nat. Protocols **2**(1): 31-34.

Girotti, A. W. (1998). "Lipid hydroperoxide generation, turnover, and effector action in biological systems." Journal of Lipid Research **39**(8): 1529-1542.

Glick, D., S. Barth and K. F. Macleod (2010). "Autophagy: cellular and molecular mechanisms." J Pathol **221**(1): 3-12.

Gorner, W., E. Durchschlag, M. T. Martinez-Pastor, F. Estruch, G. Ammerer, B. Hamilton, H. Ruis and C. Schuller (1998). "Nuclear localization of the C_2H_2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity." Genes Dev **12**(4): 586-597.

Gorner, W., E. Durchschlag, J. Wolf, E. L. Brown, G. Ammerer, H. Ruis and C. Schuller (2002). "Acute glucose starvation activates the nuclear localization signal of a stress-specific yeast transcription factor." Embo j **21**(1-2): 135-144.

Gross, E., C. S. Sevier, N. Heldman, E. Vitu, M. Bentzur, C. A. Kaiser, C. Thorpe and D. Fass (2006). "Generating disulfides enzymatically: reaction products and electron acceptors of the endoplasmic reticulum thiol oxidase Ero1p." Proc Natl Acad Sci U S A **103**(2): 299-304.

Guzy, R. D., B. Hoyos, E. Robin, H. Chen, L. Liu, K. D. Mansfield, M. C. Simon, U. Hammerling and P. T. Schumacker (2005). "Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing." Cell Metab **1**(6): 401-408.

Han, D., F. Antunes, R. Canali, D. Rettori and E. Cadenas (2003). "Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol." J Biol Chem **278**(8): 5557-5563.

Hanada, K. (2003). "Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism." Biochim Biophys Acta **1632**(1-3): 16-30.

Handy, D. E. and J. Loscalzo (2012). "Redox regulation of mitochondrial function." Antioxid Redox Signal **16**(11): 1323-1367.

Hanne;, M. and W. Xiao (2006). Isolation of Nucleic Acids. Yeast Protocols. W. Xiao. Met. Mol. Biol. **2:** 15-21.

Harju, S., H. Fedosyuk and K. Peterson (2004). "Rapid isolation of yeast genomic DNA: Bust n' Grab." BMC Biotechnology **4**(1): 1-6.

Harman, D. (1956). "Aging: a theory based on free radical and radiation chemistry." J Gerontol **11**(3): 298-300.

Harris, N., M. Bachler, V. Costa, M. Mollapour, P. Moradas-Ferreira and P. W. Piper (2005). "Overexpressed Sod1p acts either to reduce or to increase the lifespans and stress resistance of yeast, depending on whether it is Cu^{2+}-deficient or an active Cu,Zn-superoxide dismutase." Aging Cell **4**(1): 41-52.

Hayashi, N., T. Nomura, N. Sakumoto, Y. Mukai, Y. Kaneko, S. Harashima and S. Murakami (2005). "The SIT4 gene, which encodes protein phosphatase 2A, is required for telomere function in *Saccharomyces cerevisiae*." Curr Genet **47**(6): 359-367.

Hedbacker, K. and M. Carlson (2008). "SNF1/AMPK pathways in yeast." Front Biosci **13**: 2408-2420.

Hedges, D., M. Proft and K. D. Entian (1995). "CAT8, a new zinc cluster-encoding gene necessary for derepression of gluconeogenic enzymes in the yeast *Saccharomyces cerevisiae*." Mol Cell Biol **15**(4): 1915-1922.

Herrera, J. L., M. Diaz, J. R. Hernandez-Fernaud, E. Salido, R. Alonso, C. Fernandez, A. Morales and R. Marin (2011). "Voltage-dependent anion channel as a resident protein of lipid rafts: post-transductional regulation by estrogens and involvement in neuronal preservation against Alzheimer's disease." J Neurochem **116**(5): 820-827.

Hinnebusch, A. G. (2005). "Translational regulation of GCN4 and the general amino acid control of yeast." Annu Rev Microbiol **59**: 407-450.

Hong, S. P., F. C. Leiper, A. Woods, D. Carling and M. Carlson (2003). "Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases." Proc Natl Acad Sci U S A **100**(15): 8839-8843.

Hoose, S. A., J. A. Rawlings, M. M. Kelly, M. C. Leitch, Q. O. Ababneh, J. P. Robles, D. Taylor, E. M. Hoover, B. Hailu, K. A. McEnery, S. S. Downing, D. Kaushal, Y. Chen, A. Rife, K. A. Brahmbhatt, R. Smith, III and M. Polymenis (2012). "A Systematic Analysis of Cell Cycle Regulators in Yeast Reveals That Most Factors Act Independently of Cell Size to Control Initiation of Division." PLoS Genet **8**(3): e1002590.

Hoppins, S., S. R. Collins, A. Cassidy-Stone, E. Hummel, R. M. Devay, L. L. Lackner, B. Westermann, M. Schuldiner, J. S. Weissman and J. Nunnari (2011). "A mitochondrial-focused genetic interaction map reveals a scaffold-like complex required for inner membrane organization in mitochondria." J Cell Biol **195**(2): 323-340.

Huang, X., J. Liu and R. C. Dickson (2012). "Down-Regulating Sphingolipid Synthesis Increases Yeast Lifespan." PLoS Genet **8**(2): e1002493.

Huang, X., B. R. Withers and R. C. Dickson (2014). "Sphingolipids and lifespan regulation." Biochim Biophys Acta **1841**(5): 657-664.

Huh, W. K., J. V. Falvo, L. C. Gerke, A. S. Carroll, R. W. Howson, J. S. Weissman and E. K. O'Shea (2003). "Global analysis of protein localization in budding yeast." Nature **425**(6959): 686-691.

Igual, J. C., A. L. Johnson and L. H. Johnston (1996). "Coordinated regulation of gene expression by the cell cycle transcription factor Swi4 and the protein kinase C MAP kinase pathway for yeast cell integrity." Embo j **15**(18): 5001-5013.

Ishikawa, K., K. Takenaga, M. Akimoto, N. Koshikawa, A. Yamaguchi, H. Imanishi, K. Nakada, Y. Honma and J. Hayashi (2008). "ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis." Science **320**(5876): 661-664.

Jablonka, W., S. Guzman, J. Ramirez and M. Montero-Lomeli (2006). "Deviation of carbohydrate metabolism by the SIT4 phosphatase in *Saccharomyces cerevisiae*." Biochim Biophys Acta **1760**(8): 1281-1291.

Jiang, J. C., E. Jaruga, M. V. Repnevskaya and S. M. Jazwinski (2000). "An intervention resembling caloric restriction prolongs life span and retards aging in yeast." Faseb j **14**(14): 2135-2137.

Jiang, Y. and J. R. Broach (1999). "Tor proteins and protein phosphatase 2A reciprocally regulate Tap42 in controlling cell growth in yeast." Embo j **18**(10): 2782-2792.

Jin, C., A. Barrientos, C. B. Epstein, R. A. Butow and A. Tzagoloff (2007). "SIT4 regulation of Mig1p-mediated catabolite repression in *Saccharomyces cerevisiae*." FEBS Lett **581**(29): 5658-5663.

Karbowski, M. and A. Neutzner (2012). "Neurodegeneration as a consequence of failed mitochondrial maintenance." Acta Neuropathol **123**(2): 157-171.

Kerner, J., K. Lee, B. Tandler and C. L. Hoppel (2012). "VDAC proteomics: post-translation modifications." Biochim Biophys Acta **1818**(6): 1520-1525.

Ketela, T., R. Green and H. Bussey (1999). "*Saccharomyces cerevisiae* mid2p is a potential cell wall stress sensor and upstream activator of the PKC1-MPK1 cell integrity pathway." J Bacteriol **181**(11): 3330-3340.

Kitagaki, H., L. A. Cowart, N. Matmati, D. Montefusco, J. Gandy, S. V. de Avalos, S. A. Novgorodov, J. Zheng, L. M. Obeid and Y. A. Hannun (2009). "ISC1-dependent metabolic adaptation reveals an indispensable role for mitochondria in induction of nuclear genes during the diauxic shift in Saccharomyces cerevisiae." J Biol Chem **284**(16): 10818-10830.

Kitagaki, H., L. A. Cowart, N. Matmati, S. Vaena de Avalos, S. A. Novgorodov, Y. H. Zeidan, J. Bielawski, L. M. Obeid and Y. A. Hannun (2007). "Isc1 regulates sphingolipid metabolism in yeast mitochondria." Biochim Biophys Acta **1768**(11): 2849-2861.

Koopman, W. J., L. G. Nijtmans, C. E. Dieteren, P. Roestenberg, F. Valsecchi, J. A. Smeitink and P. H. Willems (2010). "Mammalian mitochondrial complex I: biogenesis, regulation, and reactive oxygen species generation." Antioxid Redox Signal **12**(12): 1431-1470.

Korshunov, S. S., B. F. Krasnikov, M. O. Pereverzev and V. P. Skulachev (1999). "The antioxidant functions of cytochrome c." FEBS Lett **462**(1-2): 192-198.

Krauss, S. (2001). Mitochondria: Structure and Role in Respiration. eLS, John Wiley & Sons, Ltd.

Kurland, C. G. and S. G. Andersson (2000). "Origin and evolution of the mitochondrial proteome." Microbiol Mol Biol Rev **64**(4): 786-820.

Kushnareva, Y., A. N. Murphy and A. Andreyev (2002). "Complex I-mediated reactive oxygen species generation: modulation by cytochrome c and NAD(P)+ oxidation-reduction state." Biochem J **368**(Pt 2): 545-553.

Kussmaul, L. and J. Hirst (2006). "The mechanism of superoxide production by NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria." Proc Natl Acad Sci U S A **103**(20): 7607-7612.

Lebovitz, R. M., H. Zhang, H. Vogel, J. Cartwright, Jr., L. Dionne, N. Lu, S. Huang and M. M. Matzuk (1996). "Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice." Proc Natl Acad Sci U S A **93**(18): 9782-9787.

Lee, A. C., X. Xu, E. Blachly-Dyson, M. Forte and M. Colombini (1998). "The role of yeast VDAC genes on the permeability of the mitochondrial outer membrane." J Membr Biol **161**(2): 173-181.

Lee, P., B. R. Cho, H. S. Joo and J. S. Hahn (2008). "Yeast Yak1 kinase, a bridge between PKA and stress-responsive transcription factors, Hsf1 and Msn2/Msn4." Mol Microbiol **70**(4): 882-895.

Lesage, P., X. Yang and M. Carlson (1996). "Yeast SNF1 protein kinase interacts with SIP4, a C6 zinc cluster transcriptional activator: a new role for SNF1 in the glucose response." Mol Cell Biol **16**(5): 1921-1928.

Levin, D. E. (2011). "Regulation of Cell Wall Biogenesis in *Saccharomyces cerevisiae:* The Cell Wall Integrity Signaling Pathway." Genetics **189**(4): 1145-1175.

Lillie, S. H. and J. R. Pringle (1980). "Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation." J Bacteriol **143**(3): 1384-1394.

Lionaki, E., M. Markaki and N. Tavernarakis (2013). "Autophagy and ageing: insights from invertebrate model organisms." Ageing Res Rev **12**(1): 413-428.

Liu, J., X. Huang, B. R. Withers, E. Blalock, K. Liu and R. C. Dickson (2013). "Reducing sphingolipid synthesis orchestrates global changes to extend yeast lifespan." Aging Cell **12**(5): 833-841.

Liu, Y., X. Xu and M. Carlson (2011). "Interaction of SNF1 protein kinase with its activating kinase Sak1." EC **10**(3): 313-319.

Loewith, R. and M. N. Hall (2011). "Target of rapamycin (TOR) in nutrient signaling and growth control." Genetics **189**(4): 1177-1201.

Longo, V. D. and P. Fabrizio (2002). "Regulation of longevity and stress resistance: a molecular strategy conserved from yeast to humans?" Cell Mol Life Sci **59**(6): 903-908.

Longo, V. D. and P. Fabrizio (2012). "Chronological Aging in *Saccharomyces cerevisiae*." Subcell Biochem **57**: 101-121.

Longo, V. D., E. B. Gralla and J. S. Valentine (1996). "Superoxide dismutase activity is essential for stationary phase survival in *Saccharomyces cerevisiae.* Mitochondrial production of toxic oxygen species in vivo." J Biol Chem **271**(21): 12275-12280.

Lorenz, D. R., C. R. Cantor and J. J. Collins (2009). "A network biology approach to aging in yeast." Proc Natl Acad Sci U S A **106**(4): 1145-1150.

Luke, M. M., F. Della Seta, C. J. Di Como, H. Sugimoto, R. Kobayashi and K. T. Arndt (1996). "The SAP, a new family of proteins, associate and function positively with the SIT4 phosphatase." Mol Cell Biol **16**(6): 2744-2755.

Mager, W. H. and J. Winderickx (2005). "Yeast as a model for medical and medicinal research." Trends Pharmacol Sci **26**(5): 265-273.

Malcher, M., S. Schladebeck and H. U. Mosch (2011). "The Yak1 protein kinase lies at the center of a regulatory cascade affecting adhesive growth and stress resistance in *Saccharomyces cerevisiae*." Genetics **187**(3): 717-730.

Mansfield, K. D., R. D. Guzy, Y. Pan, R. M. Young, T. P. Cash, P. T. Schumacker and M. C. Simon (2005). "Mitochondrial dysfunction resulting from loss of cytochrome c impairs cellular oxygen sensing and hypoxic HIF-alpha activation." Cell Metab **1**(6): 393-399.

Matmati, N. and Y. A. Hannun (2008). "Thematic review series: sphingolipids. ISC1 (inositol phosphosphingolipid-phospholipase C), the yeast homologue of neutral sphingomyelinases." J Lipid Res **49**(5): 922-928.

McCourt, P. C., J. M. Morgan and J. T. Nickels, Jr. (2009). "Stress-induced ceramide-activated protein phosphatase can compensate for loss of amphiphysin-like activity in Saccharomyces cerevisiae and functions to reinitiate endocytosis." J Biol Chem **284**(18): 11930-11941.

McDonald, B. M., M. M. Wydro, R. N. Lightowlers and J. H. Lakey (2009). "Probing the orientation of yeast VDAC1 in vivo." FEBS Lett **583**(4): 739-742.

Mesquita, A., M. Weinberger, A. Silva, B. Sampaio-Marques, B. Almeida, C. Leao, V. Costa, F. Rodrigues, W. C. Burhans and P. Ludovico (2010). "Caloric restriction or catalase inactivation extends yeast chronological lifespan by inducing H_2O_2 and superoxide dismutase activity." Proc Natl Acad Sci U S A **107**(34): 15123-15128.

Mitchell, P. and J. Moyle (1968). "Proton Translocation Coupled to ATP Hydrolysis in Rat Liver Mitochondria." Eur. J. Biochem. **4**(4): 530-539.

Montefusco, D. J., N. Matmati and Y. A. Hannun (2014). "The yeast sphingolipid signaling landscape." Chem Phys Lipids **177**: 26-40.

Mortimer, R. K. and J. R. Johnston (1959). "Life span of individual yeast cells." Nature **183**(4677): 1751-1752.

Murphy, M. P. (2009). "How mitochondria produce reactive oxygen species." Biochem J **417**(1): 1-13.

Nakamura, H., K. Nakamura and J. Yodoi (1997). "Redox regulation of cellular activation." Annu Rev Immunol **15**: 351-369.

Ocampo, A., J. Liu, E. A. Schroeder, G. S. Shadel and A. Barrientos (2012). "Mitochondrial respiratory thresholds regulate yeast chronological life span and its extension by caloric restriction." Cell Metab **16**(1): 55-67.

Ogas, J., B. J. Andrews and I. Herskowitz (1991). "Transcriptional activation of CLN1, CLN2, and a putative new G1 cyclin (HCS26) by SWI4, a positive regulator of G1-specific transcription." Cell **66**(5): 1015-1026.

Ohlmeier, S., J. K. Hiltunen and U. Bergmann (2010). "Protein phosphorylation in mitochondria – A study on fermentative and respiratory growth of *Saccharomyces cerevisiae*." Electrophoresis **31**(17): 2869-2881.

Okado-Matsumoto, A. and I. Fridovich (2001). "Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu,Zn-SOD in mitochondria." J Biol Chem **276**(42): 38388-38393.

Okamoto, K., P. S. Perlman and R. A. Butow (1998). "The sorting of mitochondrial DNA and mitochondrial proteins in zygotes: preferential transmission of mitochondrial DNA to the medial bud." J Cell Biol **142**(3): 613-623.

Pan, Y. (2011). "Mitochondria, reactive oxygen species, and chronological aging: a message from yeast." Exp Gerontol **46**(11): 847-852.

Pan, Y., E. A. Schroeder, A. Ocampo, A. Barrientos and G. S. Shadel (2011). "Regulation of yeast chronological life span by TORC1 via adaptive mitochondrial ROS signaling." Cell Metab **13**(6): 668-678.

Panfili, E., G. Sandri and L. Ernster (1991). "Distribution of glutathione peroxidases and glutathione reductase in rat brain mitochondria." FEBS Lett **290**(1-2): 35-37.

Pearce, L. R., D. Komander and D. R. Alessi (2010). "The nuts and bolts of AGC protein kinases." Nat Rev Mol Cell Biol **11**(1): 9-22.

Pereira, C., N. Camougrand, S. Manon, M. J. Sousa and M. Corte-Real (2007). "ADP/ATP carrier is required for mitochondrial outer membrane permeabilization and cytochrome c release in yeast apoptosis." Mol Microbiol **66**(3): 571-582.

Poppek, D. and T. Grune (2006). "Proteasomal defense of oxidative protein modifications." Antioxid Redox Signal **8**(1-2): 173-184.

Powers, R. W., 3rd, M. Kaeberlein, S. D. Caldwell, B. K. Kennedy and S. Fields (2006). "Extension of chronological life span in yeast by decreased TOR pathway signaling." Genes Dev **20**(2): 174-184.

Powers, T. (2007). "TOR signaling and S6 kinase 1: Yeast catches up." Cell Metab **6**(1): 1-2.

Qi, Y., X. Tian, J. Liu, Y. Han, A. M. Graham, M. C. Simon, J. M. Penninger, P. Carmeliet and S. Li (2012). "Bnip3 and AIF cooperate to induce apoptosis and cavitation during epithelial morphogenesis." J Cell Biol **198**(1): 103-114.

Reeve, A. K., K. J. Krishnan and D. M. Turnbull (2008). "Age related mitochondrial degenerative disorders in humans." Biotechnol J **3**(6): 750-756.

Rego, A., D. Trindade, S. R. Chaves, S. Manon, V. Costa, M. J. Sousa and M. Corte-Real (2013). "The yeast model system as a tool towards the understanding of apoptosis regulation by sphingolipids." FEMS Yeast Res.

Reinders, A., N. Burckert, T. Boller, A. Wiemken and C. De Virgilio (1998). "Saccharomyces cerevisiae cAMP-dependent protein kinase controls entry into stationary phase through the Rim15p protein kinase." Genes Dev **12**(18): 2943-2955.

Rhee, S. G., H. Z. Chae and K. Kim (2005). "Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling." Free Radic Biol Med **38**(12): 1543-1552.

Ristow, M. and S. Schmeisser (2011). "Extending life span by increasing oxidative stress." Free Radic Biol Med **51**(2): 327-336.

Roosen, J., K. Engelen, K. Marchal, J. Mathys, G. Griffioen, E. Cameroni, J. M. Thevelein, C. De Virgilio, B. De Moor and J. Winderickx (2005). "PKA and Sch9 control a molecular switch important for the proper adaptation to nutrient availability." Mol Microbiol **55**(3): 862-880.

Rostovtseva, T. and M. Colombini (1997). "VDAC channels mediate and gate the flow of ATP: implications for the regulation of mitochondrial function." Biophys J **72**(5): 1954-1962.

Ruiz, A., X. Xu and M. Carlson (2011). "Roles of two protein phosphatases, Reg1-Glc7 and Sit4, and glycogen synthesis in regulation of SNF1 protein kinase." Proc Natl Acad Sci U S A **108**(16): 6349-6354.

Salvi, M., V. Battaglia, A. M. Brunati, N. La Rocca, E. Tibaldi, P. Pietrangeli, L. Marcocci, B. Mondovi, C. A. Rossi and A. Toninello (2007). "Catalase takes part in rat liver mitochondria oxidative stress defense." J Biol Chem **282**(33): 24407-24415.

Sanchez, N. S., D. A. Pearce, T. S. Cardillo, S. Uribe and F. Sherman (2001). "Requirements of Cyc2p and the porin, Por1p, for ionic stability and mitochondrial integrity in *Saccharomyces cerevisiae*." Arch Biochem Biophys **392**(2): 326-332.

Saretzki, G., L. Armstrong, A. Leake, M. Lako and T. von Zglinicki (2004). "Stress defense in murine embryonic stem cells is superior to that of various differentiated murine cells." Stem Cells **22**(6): 962-971.

Scherz-Shouval, R., E. Shvets, E. Fass, H. Shorer, L. Gil and Z. Elazar (2007). "Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4." Embo j **26**(7): 1749-1760.

Schmelzle, T., S. B. Helliwell and M. N. Hall (2002). "Yeast protein kinases and the RHO1 exchange factor TUS1 are novel components of the cell integrity pathway in yeast." Mol Cell Biol **22**(5): 1329-1339.

Schoppink, P. J., W. Hemrika and J. A. Berden (1989). "The effect of deletion of the genes encoding the 40 kDa subunit II or the 17 kDa subunit VI on the steady-state kinetics of yeast ubiquinol—cytochrome-c oxidoreductase." Biochimica et Biophysica Acta (BBA) - Bioenergetics **974**(2): 192-201.

Schroeder, E. A. and G. S. Shadel (2014). "Crosstalk between mitochondrial stress signals regulates yeast chronological lifespan." Mech Ageing Dev **135**: 41-49.

Sena, L. A. and N. S. Chandel (2012). "Physiological roles of mitochondrial reactive oxygen species." Mol Cell **48**(2): 158-167.

Sheldon, K. L., E. N. Maldonado, J. J. Lemasters, T. K. Rostovtseva and S. M. Bezrukov (2011). "Phosphorylation of Voltage-Dependent Anion Channel by Serine/Threonine Kinases Governs Its Interaction with Tubulin." PLoS ONE **6**(10): e25539.

Silva, R. D., L. Saraiva, I. Coutinho, J. Goncalves and M. Corte-Real (2012). "Yeast as a powerful model system for the study of apoptosis regulation by protein kinase C isoforms." Curr Pharm Des **18**(17): 2492-2500.

Singer, T., S. Haefner, M. Hoffmann, M. Fischer, J. Ilyina and W. Hilt (2003). "Sit4 phosphatase is functionally linked to the ubiquitin-proteasome system." Genetics **164**(4): 1305-1321.

Smets, B., R. Ghillebert, P. De Snijder, M. Binda, E. Swinnen, C. De Virgilio and J. Winderickx (2010). "Life in the midst of scarcity: adaptations to nutrient availability in *Saccharomyces cerevisiae*." Curr Genet **56**(1): 1-32.

Smith, D. L., Jr., J. M. McClure, M. Matecic and J. S. Smith (2007). "Calorie restriction extends the chronological lifespan of Saccharomyces cerevisiae independently of the Sirtuins." Aging Cell **6**(5): 649-662.

Smith, P. M., J. L. Fox and D. R. Winge (2012). "Biogenesis of the cytochrome bc(1) complex and role of assembly factors." Biochim Biophys Acta **1817**(2): 276-286.

Spincemaille, P., N. Matmati, Y. A. Hannun, B. P. Cammue and K. Thevissen (2014). "Sphingolipids and mitochondrial function in budding yeast." Biochim Biophys Acta **1840**(10): 3131-3137.

Stadtman, E. R. (2004). "Role of oxidant species in aging." Curr Med Chem **11**(9): 1105-1112.

Stewart, V. C., M. A. Sharpe, J. B. Clark and S. J. Heales (2000). "Astrocyte-derived nitric oxide causes both reversible and irreversible damage to the neuronal mitochondrial respiratory chain." J Neurochem **75**(2): 694-700.

Strogolova, V., M. Orlova, A. Shevade and S. Kuchin (2012). "Mitochondrial Porin Por1 and Its Homolog Por2 Contribute to the Positive Control of Snf1 Protein Kinase in *Saccharomyces cerevisiae*." EC **11**(12): 1568-1572.

Susin, S. A., H. K. Lorenzo, N. Zamzami, I. Marzo, B. E. Snow, G. M. Brothers, J. Mangion, E. Jacotot, P. Costantini, M. Loeffler, N. Larochette, D. R. Goodlett, R. Aebersold, D. P. Siderovski, J. M. Penninger and G. Kroemer (1999). "Molecular characterization of mitochondrial apoptosis-inducing factor." Nature **397**(6718): 441-446.

Sutherland, C. M., S. A. Hawley, R. R. McCartney, A. Leech, M. J. R. Stark, M. C. Schmidt and D. G. Hardie (2003). "Elm1p Is One of Three Upstream Kinases for the *Saccharomyces cerevisiae* SNF1 Complex." Current Biology **13**(15): 1299-1305.

Sutton, A., D. Immanuel and K. T. Arndt (1991). "The SIT4 protein phosphatase functions in late G1 for progression into S phase." Mol Biol Cell **11**(4): 2133-2148.

Swinnen, E., T. Wilms, J. Idkowiak-Baldys, B. Smets, P. De Snijder, S. Accardo, R. Ghillebert, K. Thevissen, B. Cammue, D. De Vos, J. Bielawski, Y. A. Hannun and J. Winderickx (2014). "The protein kinase Sch9 is a key regulator of sphingolipid metabolism in *Saccharomyces cerevisiae*." Mol Biol Cell **25**(1): 196-211.

Tate, J. J., I. Georis, E. Dubois and T. G. Cooper (2010). "Distinct Phosphatase Requirements and GATA Factor Responses to Nitrogen Catabolite Repression and Rapamycin Treatment in *Saccharomyces cerevisiae*." J Biol Chem **285**(23): 17880-17895.

Teixeira, V., T. C. Medeiros, R. Vilaça, P. Moradas-Ferreira and V. Costa (2014). "Reduced TORC1 signaling abolishes mitochondrial dysfunctions and shortened chronological lifespan of Isc1p-deficient cells." Microbial Cell 1(1): 21-36.

Thorpe, G. W., M. Reodica, M. J. Davies, G. Heeren, S. Jarolim, B. Pillay, M. Breitenbach, V. J. Higgins and I. W. Dawes (2013). "Superoxide radicals have a protective role during H_2O_2 stress." Mol Biol Cell **24**(18): 2876-2884.

Tormos, K. V., E. Anso, R. B. Hamanaka, J. Eisenbart, J. Joseph, B. Kalyanaraman and N. S. Chandel (2011). "Mitochondrial complex III ROS regulate adipocyte differentiation." Cell Metab **14**(4): 537-544.

Trachootham, D., W. Lu, M. A. Ogasawara, R. D. Nilsa and P. Huang (2008). "Redox regulation of cell survival." Antioxid Redox Signal **10**(8): 1343-1374.

Vaena de Avalos, S., Y. Okamoto and Y. A. Hannun (2004). "Activation and localization of inositol phosphosphingolipid phospholipase C, Isc1p, to the mitochondria during growth of *Saccharomyces cerevisiae*." J Biol Chem **279**(12): 11537-11545.

Valko, M., C. J. Rhodes, J. Moncol, M. Izakovic and M. Mazur (2006). "Free radicals, metals and antioxidants in oxidative stress-induced cancer." Chem Biol Interact **160**(1): 1-40.

Venditti, P., L. Di Stefano and S. Di Meo (2013). "Mitochondrial metabolism of reactive oxygen species." Mitochondrion **13**(2): 71-82.

Vilaca, R., E. Silva, A. Nadais, V. Teixeira, N. Matmati, J. Gaifem, Y. A. Hannun, M. C. Sa Miranda and V. Costa (2014). "Sphingolipid signalling mediates mitochondrial dysfunctions and reduced chronological lifespan in the yeast model of Niemann-Pick type C1." Mol Microbiol **91**(3): 438-451.

Wink, D. A., R. W. Nims, J. F. Darbyshire, D. Christodoulou, I. Hanbauer, G. W. Cox, F. Laval, J. Laval, J. A. Cook, M. C. Krishna and et al. (1994). "Reaction kinetics for nitrosation of cysteine and glutathione in aerobic nitric oxide solutions at neutral pH. Insights into the fate and physiological effects of intermediates generated in the NO/O_2 reaction." Chem Res Toxicol **7**(4): 519-525.

Winterbourn, C. C. (2008). "Reconciling the chemistry and biology of reactive oxygen species." Nat Chem Biol **4**(5): 278-286.

Woodacre, A., M. A. Lone, D. Jablonowski, R. Schneiter, F. Giorgini and R. Schaffrath (2013). "A novel Sit4 phosphatase complex is involved in the response to ceramide stress in yeast." Oxid Med Cell Longev **2013**: 129645.

Yamada, Y. and H. Harashima (2008). "Mitochondrial drug delivery systems for macromolecule and their therapeutic application to mitochondrial diseases." Adv Drug Deliv Rev **60**(13-14): 1439-1462.

Yang, R., K. T. Chun and R. C. Wek (1998). "Mitochondrial Respiratory Mutants in Yeast Inhibit Glycogen Accumulation by Blocking Activation of Glycogen Synthase." J Biol Chem **273**(47): 31337-31344.

Young, E. T., K. M. Dombek, C. Tachibana and T. Ideker (2003). "Multiple pathways are co-regulated by the protein kinase Snf1 and the transcription factors Adr1 and Cat8." J Biol Chem **278**(28): 26146-26158.

Zara, V., L. Conte and B. L. Trumpower (2007). "Identification and characterization of cytochrome bc(1) subcomplexes in mitochondria from yeast with single and double deletions of genes encoding cytochrome bc(1) subunits." Febs j **274**(17): 4526-4539.

Zarzov, P., C. Mazzoni and C. Mann (1996). "The SLT2(MPK1) MAP kinase is activated during periods of polarized cell growth in yeast." Embo j **15**(1): 83-91.

Zheng, Y. and Y. Jiang (2005). "The yeast phosphotyrosyl phosphatase activator is part of the Tap42-phosphatase complexes." Mol Biol Cell **16**(4): 2119-2127.

CHAPTER 8
ATTACHMENTS

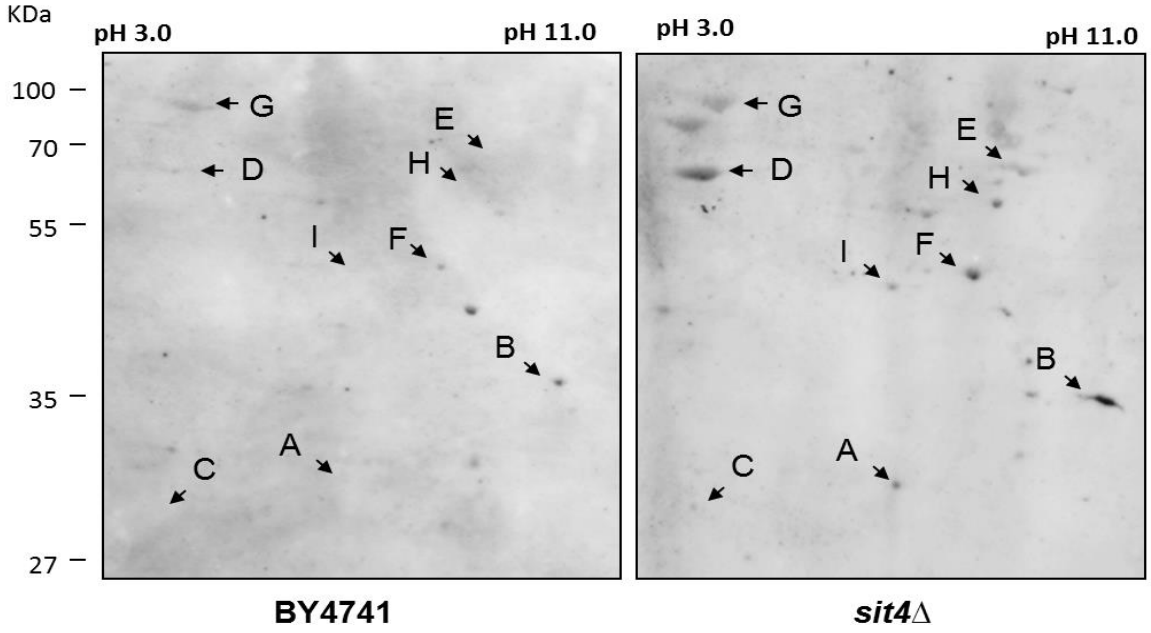

Figure S1 - Analysis of changes in the mitochondrial phosphoproteome of *sit4*Δ cells. Mitochondria were isolated from WT and *sit4*Δ cells and proteins were separated by two-dimensional gel electrophoresis and blotted into a nitrocellulose membrane. Protein phosphorylation was analysed by western blot, using an antibody that recognizes phosphorylated residues. Proteins differentially phosphorylated in *sit4*Δ cells were identified by mass spectrometry (MALDI-MS/MS), using spots cut from a silver stained 2D-gel replicate. The identification of proteins differentially phosphorylated in *sit4*Δ cells (A-I) is shown in Table S1.

Table S1 - Identification and function of the hyperphosphorylated mitochondrial proteins in *sit4*Δ cells.

Hyperphosphorylated proteins		
Spot	Protein name	Protein Function
A	Sod2p	Superoxide dismutase [Mn]
B	Por1p	Mitochondrial outer membrane protein
C	Hsp26p	Heat shock protein 26
D	Atp2p	ATP synthase subunit beta
E	Atp1p	ATP synthase subunit alpha
F	Ilv5p	Ketol-acid reductoisomerase
G	Ssc1p	Heat shock protein SSC1
H	Pdip	Protein disulfide-isomerase
I	Qcr2p	Cytochrome b-c1 complex subunit 2

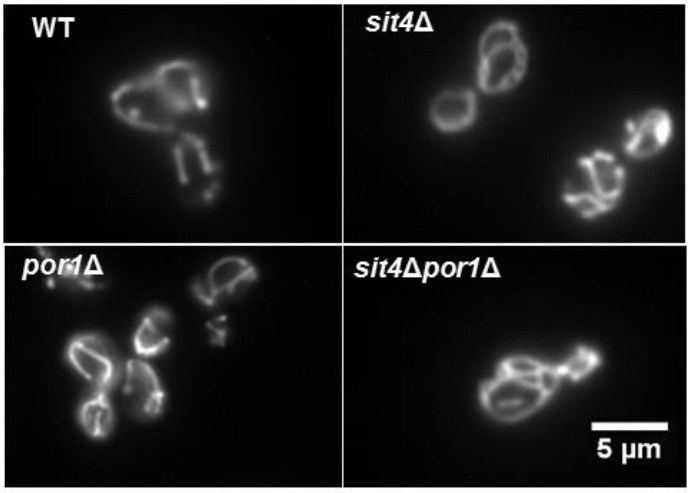

Figure S2 - Mitochondrial network morphology. The mitochondrial network was visualized in each mutant by using a matrix-targeted GFP. The cells were grown in YPGal medium and were analysed using an AxioImager Z1 (Carl Zeiss) fluorescence microscope.